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**THE AMERICAN  
UNIVERSITY IN CAIRO**  
الجامعة الأمريكية بالقاهرة

**School of Sciences and Engineering**

**Impacts of prolonged exposure to low concentration of Titanium dioxide nanoparticles on cell cycle control and DNA repair in Normal, Cancer and DNA repair deficient cells.**

**A Thesis Submitted to**

**Biotechnology**

**In partial fulfillment of the requirements for the degree of Master of Science.**

**by Nada Hazem EL Zahed.**

**(under the supervision of Dr. Andreas Kakarougkas)**

**February 2018**

## **Acknowledgments**

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**The American University in Cairo**

**Impacts of prolonged exposure to low concentration of Titanium dioxide nanoparticles on cell cycle control and DNA repair in Normal, Cancer and DNA repair deficient cells.**

**Nada Hazem Elzahed**

**Dr. Andreas Kakaroukas**

**ABSTRACT**

Nowadays, Titanium dioxide nanoparticles, TiO<sub>2</sub> NPs, are produced in huge quantities due to their vast range of applications including paints, food coloring, sunscreens and cosmetics. Consequently, humans are exposed to TiO<sub>2</sub> NPs on a daily basis. However, the toxicological profile of TiO<sub>2</sub> NPs is not fully elucidated. As a result, this study is carried out to evaluate the genotoxic impact of TiO<sub>2</sub> NPs on normal, cancer and DNA repair deficient cells. Since most of the studies evaluated the genotoxic impact of the TiO<sub>2</sub> NPs have used the acute exposure scenario: High exposure concentrations and short exposure times, in the present study the genotoxic impact of the NPs would be evaluated using the prolonged exposure scenario: low exposure concentration for long exposure time.

Cytotoxicity on a cancer cell line was evaluated using the MTT assay, genotoxicity on normal and cancer cell line was evaluated using immunofluorescent staining for anti- $\gamma$ -H2AX and anti-total-53BP1, and the impact of the NPs on cell cycle regulation was evaluated using G2/M checkpoint assay on normal and cancer cell lines. In order to study the impact of NPs on DNA repair genotoxicity was evaluated using immunofluorescent staining for anti- $\gamma$ -H2AX and anti-total-53BP1 on normal and DNA repair deficient cell lines. Finally, the maintenance of cell cycle G2/M checkpoint in absence of DNA repair genes was evaluated using the G2/M checkpoint assay on DNA repair deficient cell lines.

In the present study, we have demonstrated that long exposures to TiO<sub>2</sub> NPs does not induces cytotoxicity but it causes significant genotoxicity, particularly DNA double strand breaks. More precisely, we verified that NPs induces DNA DSBs at heterochromatin as well as euchromatin regions of the genome. Furthermore, we demonstrated that DNA DSBs repair, during G1 phase, at heterochromatin region is ATM dependent while DNA DSBs repair at Euchromatin regions is ATM independent, and DNA PKcs and Artemis dependent. On the other hand, it was seen that activation of G2/M cell cycle checkpoint after exposure to the NPs has DNA DSBs dependent-threshold. Also, it was shown that the release of the cell cycle checkpoint has DNA DSBs dependent-threshold. Lastly, we observed cell cycle checkpoint adaptation on prolonged exposure scenario. Taken together, we have demonstrated that prolonged exposure scenario does not affect cell viability but it causes DNA damage and cell cycle checkpoint adaptation leading to genetic instability.

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### List of Acronyms Justify the text of the list of abbreviations

• TiO <sub>2</sub> NPs	Titanium dioxide Nanoparticles
• FAO	Food and agriculture organization
• WHO	World Health Organization
• FDA	Food and Drug administration
• ROS	Reactive oxygen species
• DNA	Deoxyribonucleic acid
• DSBs	Double strand breaks
• SSB	Single strand break
• BER	Base excision repair
• NER	Nucleotide excision repair
• MMR	Mismatch repair
• c-NHEJ	Classical non homologues end joining
• HRR	Homologous recombination repair
• alt-EJ	Alternative end joining
• NCS	Neocarzinostatin
• DMEM	Dulbecco's modified essential medium
• DMSO	Dimethyl sulfoxate
• FBS	Fetal bovine serum
• FITC	Fluorescein Iso-thiocyanate
• TRITC	Tetramethyl Rhodamine Iso-thiocyanate
• µg	Microgram
• ml	Milliliter
• MEFs	Mouse embryonic fibroblasts
• BSA	Bovine serum Albumin
• MTT	3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromide



## 1. Introduction

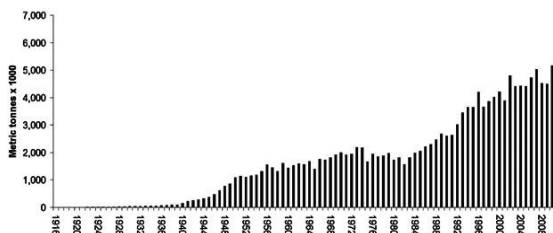
### 1.1 Cancer and Titanium dioxide Nanoparticles

#### 1.1.1 Titanium dioxide Nanoparticles.

Due to the massive expansion in nanotechnology there are lots of daily life applications includes nanomaterial such as drugs, food and pharmaceutical products (Emerich et al. 2013). For the vast range of applications of TiO<sub>2</sub> NPs, titanium dioxide nanoparticles, in everyday products, TiO<sub>2</sub> NPs has become one of the widely produced nanoparticles. It was discovered that titanium dioxide improves the brightness and whiteness of products, opacity and resists decolorization (Skocaj, Filipic, Petkovic, & Novak, 2011). For these reasons, and beside its low price as a raw material, it is regularly used in food additives, toothpastes, cosmetics, creams and paints (Shi, Magaye, Castranova, & Zhao, 2013). Moreover it was shown that it has the ability to reflect UV light. As a result, it is added as a main ingredient in most of the sunscreens (Zhang et al, 2015). All of the uses mentioned above clearly show why millions of tons of titanium dioxide are produced every year.

In 1969, The Joint FAO/WHO Expert Committee on Food additives carried out the first toxicological evaluation of TiO<sub>2</sub> NPs (JECFA1969), proving that titanium dioxide is a very insoluble compound and shows neither significance tissue absorption nor storage. For that reason, titanium dioxide was considered to be biologically inert in human tissues (Skocaj et al., 2011) and not to cause a severe lethality (Johnston et al., 2009). As a result, it was added by the United States in the Food and Drug Administration (FDA) as Inactive Ingredients (Skocaj et al., 2011) and by European Union (FAO/WHO 2010) as a food additive primarily used in variety of consumer products.

The approval of titanium dioxide as food additive by U.S and EU, and the vast range of applications that includes titanium dioxide nanoparticles, resulted in historical production of TiO<sub>2</sub> nanoparticles to reach a total of 165050000 metric tons from 1916 to 2011 in worldwide (Jovanović, 2015). Thus, titanium dioxide has become one of the top 5 nanoparticles used in consumer products (Rollerova et al, 2015). Eventually, concerns rose about TiO<sub>2</sub> NPs' impact on human health. This resulted in invitro and invivo studies that were performed to assess the toxicity of these nanoparticles that may affect human health. Consequently, results will provide the regulatory agencies with a reliable data to reassess the risk-benefit ratio of TiO<sub>2</sub> particles.



**Figure 1: Historical production of Titanium dioxide nanoparticles worldwide, (Jovanović, 2015).**

Most of the results of invitro and invivo studies makes these nanoparticles experimentally categorized as “possible carcinogenic to humans” and as” occupational carcinogen” by the International Agency for Research on Cancer and the National Institute for Occupational Safety and Health, respectively. This is because most of the studies concluded that following the treatment of the cells with TiO<sub>2</sub> NPs results in impairment to major cellular components such as lipids, protein, DNA and chromosomes (Gurr, Wang, Chen, & Jan, 2005) (Park et al., 2008). Moreover, it results in nuclear fragmentation, caspases activation, and cell death through apoptosis or necrosis (Gurr et al., 2005; Kang, Kim, Lee, & Chung, 2008) (Park et al., 2008).

However, recent studies showed evidence of cell resistance to TiO<sub>2</sub> nanoparticle toxicity (Singh et al., 2007) and this was further confirmed by recent studies, showing that TiO<sub>2</sub> NPs’ toxicity is affected by the physiochemical properties of TiO<sub>2</sub> NPs and the origin of cells studied. This raises a question, if titanium dioxide toxicity can be managed to stimulate normal cell resistance and cancer cell sensitivity so that it can be used as an anticancer agent? To answer this question, a recent study was performed to show that TiO<sub>2</sub> NPs can results in cellular death of transformed cells (Zhu, Eaton, & Li, 2012). This shows the importance of further studies to be performed to study the toxicity of titanium dioxide nanoparticle on cancer cell as well as normal cell. Thus, new strategies will arise to use titanium dioxide nanoparticles as a new anti-cancer agent.

### **1.1.2 Carcinogenicity of Titanium dioxide Nanoparticles.**

#### **On Skin**

Skin cancer has become frequent in recent decades (Nohynek & Schaefer, 2001). This is because skin is the first organ any particle in the surrounding environment can come into direct contact. As a consequence the use of protective sunscreens becomes a trend among the population. But actually, as it was mentioned above, most of the sunscreens found to have titanium dioxide nanoparticles as one of the main ingredients which serves as one of the main routes for the TiO<sub>2</sub> to penetrate through skin to reach other body organs. For that reason, Experiments were performed to observe toxicity of TiO<sub>2</sub> NPs on skin cells. One of the most popular skin cell line used in invitro studies is Human Keratinocyte (HaCat). Some studies reported the exposure of titanium dioxide nanoparticle to HaCat cells induces phototoxicity through generation of reactive oxygen species after irradiation with UVA (Yin et al., 2012). Others, showed cellular stress activation and metabolic capacity reduction (Tucci et al., 2013). Also, the penetration ability and the possible toxicity of TiO<sub>2</sub> NPs were shown invivo via dermal route, concluding that TiO<sub>2</sub> can penetrate through skin and causes severe damage to liver. This was evidence that the dermal exposure will not induce skin toxicity only but other serious organs toxicity(Wu et al., 2009).

## **On Respiratory system**

During production, distribution and use of titanium dioxide nanoparticles it is easy for the particles to spread in the air. Thus, inhalation of titanium dioxide nanoparticles is very common. Since the primary target organ system for the inhaled particles is the respiratory system many studies were carried out using human lung cell line to examine TiO<sub>2</sub> toxicity. Most of them used A549, Adenocarcinomic human alveolar basal epithelial cells, to study the toxicity of titanium dioxide nanoparticles. One study reported induced cellular apoptosis, oxidative damage, and genotoxicity. Also, it was found alteration of apoptotic markers' expression which was a good correlation with the apoptotic/ genotoxic events expressed in the cells (Srivastava et al., 2013). Similar study showed that TiO<sub>2</sub> NP were genotoxic and induces oxidative damage in A549 cell line. Also, it was shown that DNA damage exerted by TiO<sub>2</sub> NP was not DSBs nor chromosomal damage but it was SSB, single strand break. Moreover, they observed that the NPs inhibits DNA repair through inhibition of NER and BER (Jugan et al., 2012). Two more recent papers concluded the same conclusions mentioned above that the nanoparticle inhibits proliferation and causes DNA damage. Moreover they observed that TiO<sub>2</sub> NPs results in cellular apoptosis through Mitochondrion mediated way (Wang, Sanderson, & Wang, 2007). Although many studies showed induced DNA single strand breaks via ROS formation one study showed a contradictory results and observed DNA double strands damage that was exerted directly by the nanoparticles on the DNA (Toyooka, Amano, & Ibuki, 2012). Another pulmonary cell line H1299 was tested for the toxicity after exposure to titanium dioxide nanoparticles concluding that it was more sensitive than A<sub>549</sub> because it has higher capacity to ingest the nanoparticles (Tedja et al. 2011). This clearly shows that the toxicity of the nanoparticle is affected by the cell type and concentration used.

## **On kidney and liver**

Once titanium dioxide enters human body it is widely distributed all over the body. One of the major distributed sites is liver and followed by kidney. So scientists paid attention to these two organs and a lot of studies were performed to evaluate titanium dioxide nanoparticle toxicity on liver and kidney. One study concluded that titanium dioxide nanoparticle induces DNA breaks in HepG2 cell line (Shukla et al., 2013). Similar results were shown by Prasad et al, in addition to DNA breaks that were observed using different techniques such as comet and micronucleus assays. Another study concluded more explained results showing the generation of Reactive oxygen species and induction of cellular apoptosis (El-Said, Ali, Kanehira, & Taniguchi, 2014). They explained that upon exposure to the NPs, the production Hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>, and OH increases resulting in DNA damage, activation of p53 and induction of apoptosis. Moreover, induction of ATM and IP6K3, DNA damage marker genes, showing that the damage is due to DSBs, condensation of chromatin, nuclear fragmentation and cellular apoptosis. Human embryonic kidney cells (HEK-293) were used to evaluate the toxicity of titanium dioxide nanoparticle on kidney. It was observed that TiO<sub>2</sub> anatase inhibited the division of HEK-293 cells by causing time and dose dependent cellular apoptosis.

Moreover it was observed that TiO<sub>2</sub> NPs induces the up regulation of caspase 3 as well as BAX (Meena, Rani, Pal, & Rajamani, 2012).

### **1.1.3 Anticarcinogenicity of Titanium dioxide Nanoparticles.**

Although Titanium dioxide was observed in many studies that it causes toxicity in terms of DNA damage which leads to Cancer, recently several studies observed that titanium dioxide nanoparticle can be used to treat cancer rather than inducing it. This is why titanium dioxide nanoparticle had received attention nowadays for their use in cancer therapy. In some cases the anticancer activity may result from the nanoparticle itself or in combination with other therapies (Vinardell & Mitjans, 2015). It was shown that TiO<sub>2</sub> NPs have the ability kill cancer cells. This is because titanium dioxide has a photo catalytic activity that could eradicate cancer cell with UV irradiation (Cui et al., 2013). The need of UV light has two limitations for the use of TiO<sub>2</sub> as anticancer therapy. First it should be directed towards the cancer tissue and UV light has limited penetration ability so this kind of treatment will be restricted to surface cancerous tissues (Cui et al., 2013). Thus, other studies explained the necessity for surface treatment of TiO<sub>2</sub> NP to increase its penetration and target the cancer cells (Vinardell & Mitjans, 2015). Furthermore, inhibition of proliferation of osteosarcoma cell lines was observed suggesting that titanium dioxide should be further investigated for the use of anticancer therapy (Chatterjee et al. 2016).

#### **1.1.4 Tackling of Previous studies limitations.**

As mentioned above titanium dioxide is one of the top 5 nanoparticles produced nowadays. Thus, it was intensively studied to evaluate the NPs' toxicity. However completely different conclusions were shown from several studies. Thirty four years ago, the international Program on chemical safety (1982) showed the ingested titanium dioxide is mostly excreted in urine and not stored in human body. Moreover, some recent papers showed no evidence that TiO<sub>2</sub> NP induces genotoxic or phototoxic effects to humans however it was shown that the NPs can protect subjects against skin cancer (Schilling et al., 2010). Others showed TiO<sub>2</sub> NP can preferentially results in transformed cellular death (Zhu et al., 2012). However, other recent studies concluded that titanium dioxide nanoparticles has the potential risk to induce cytotoxicity, genotoxicity, phototoxicity and oxidative stress on different organs such as Lungs, Skin, Brain, Liver & Kidney. This clearly shows that there is a huge gap remained unaddressed in this area and should be further studied critically to safely use these nanoparticles to protect consumers as well as workers.

One of the main limitations in the studies performed on evaluating the toxicity of TiO<sub>2</sub> NPs is that most of the studies focus on the molecular mechanism of inducing DNA damage only. Although studying the molecular mechanisms of inducing DNA damage is critical to evaluate the toxicity of NPs, it is essential to find out the molecular mechanism of inhibiting DNA repair pathways which may results in cellular death after exposure to titanium dioxide nanoparticles. This is because it was observed in one study, after exposure of cells to TiO<sub>2</sub> NPs DNA repair through BER and NER pathways is impaired (Jugan et al., 2012). Another study shows evidence that one of the main reasons for cell death after treatment with TiO<sub>2</sub> NPs is the down regulation of DNA repair genes. This reveals that titanium dioxide NPs have great impacts on DNA repair processes. Therefore, more studies are needed to explain the role of different proteins needed for DNA repair processes that are affected by the exposure to titanium dioxide nanoparticles leading to cellular death.

Another limitation is that most of the studies used unrealistic scenarios to assess titanium dioxide nanoparticle's toxicity using wide varieties of cell lines. This is because authors use to expose cell lines for a very high concentrations of titanium dioxide, between 5 and 200 micrograms per ml, for short exposure times, less than 24 hours (Chen et al, 2014). These concentrations were shown to be 10<sup>6</sup> higher than inhalation of humans to very high concentration of TiO<sub>2</sub> NPs after exposure on their worst-case scenario (Paul et al, 2011) or even higher than the concentrations where subjects could exposed to during their whole life (Weir, Westerhoff, Fabricius, Hristovski, & von Goetz, 2012). Thus, further studies using more realistic scenarios are needed as the biological effects and cellular toxicity differ significantly depending on exposure concentration and time.

To fill the gaps remaining unaddressed, all the possible mechanisms of inducing toxicity by NPs will be investigated in this study. This will be achieved by studying DNA damage, repair of DNA damage, and control of the cell cycle through checkpoint activation in normal, and DNA repair deficient cell lines exposed to TiO<sub>2</sub> NPs. Most importantly, very low concentrations of NPs will be used in this study to represent the real situation for Humans. Finally to highlight the possibility of manipulating the toxicity of these NPs to kill cancer cells rather than causing carcinogenicity, toxicological profile of these nanoparticles will be performed on normal vs. cancer cells.

## **1.2 DNA damage and repair**

### **1.2.1 Titanium dioxide nanoparticles and DNA lesions**

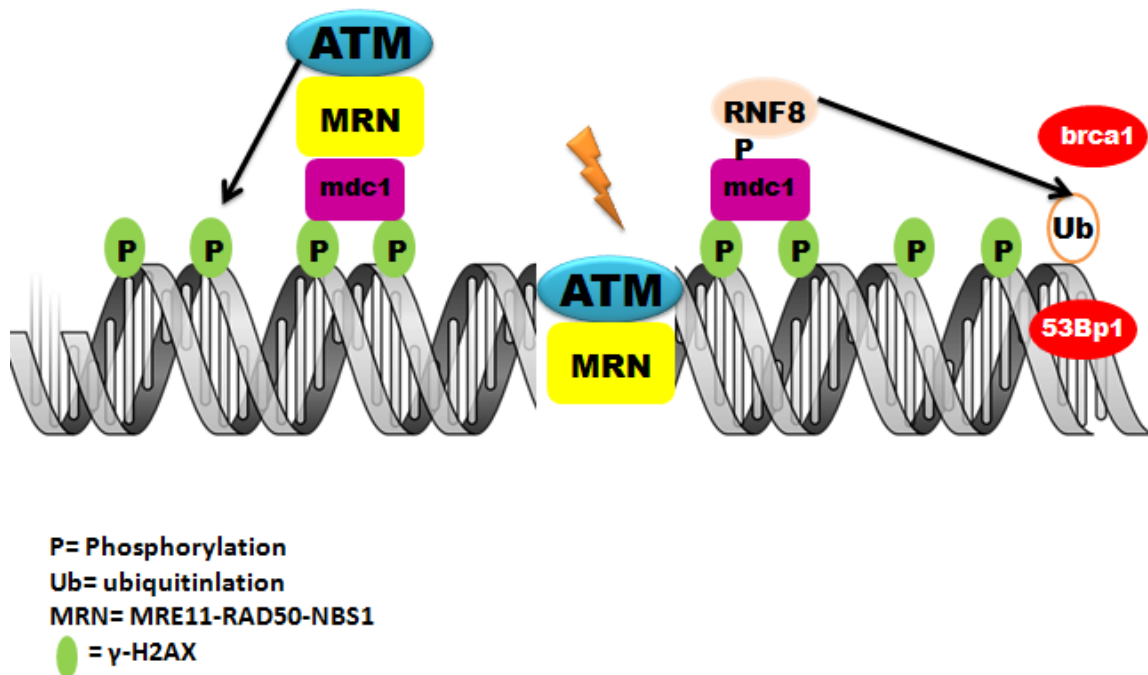
Our cells are constantly insulted by extrinsic or intrinsic DNA destructive elements such as external UV light or internal ROS produced from cellular metabolism, respectively. There are several ways by which DNA damaging agents can induce DNA damage or lesions. These lesions could be modifications in nitrogen bases or sugars, cross links, base-free sites, single or double strand breaks (Dexheimer, 2013). DNA double strand breaks (DSBs) are the most biologically dangerous kind of DNA damage. This is because single Double strand breaks can cause complete cellular death or genomic instability that can leads to carcinogenesis (Dexheimer, 2013). Many studies shows contradictory results in different types of DNA damage resulted from the NPs either single or double strand breaks. This is because the type of DNA damage induced by Titanium dioxide nanoparticles can be different according to the characteristics of the nanoparticles used in each study. In this study, TiO<sub>2</sub> NPs were found to induce one of the hazardous types of DNA damage which is DNA DSBs. Thus, DSBs are of particular interest in this study.

### **1.2.2 DNA repair pathways**

In order to compensate for the varieties of DNA damage that can occur within the cell, several DNA repair mechanisms correct DNA lesions to maintain genetic stability and prevent carcinogenesis. These different DNA repair pathways includes: Base excision repair (BER), Nucleotide excision repair (NER), Mismatch repair (MMR) and Double strand breaks repair (DNA DSBR) (Mathwes et al. 2013). Since DNA double strand break are of particular interest in this study, different DNA double strand breaks repair mechanisms will be studied.

### 1.2.3 Chromatin remodeling for double strand breaks repair.

Chromatin remodeling occurs upon induction of double strand breaks to facilitate DNA DSBs repair. This is because the induced DNA DSBs recruits sensor proteins, the MRN complex, mre11, rad50 and nbs1 proteins, towards the breaks in order to facilitate the recruitments and activation of ATM protein kinase (Lavin, 2008) (Sun, Jiang, & Price, 2010). Upon activation of ATM hundreds of proteins are phosphorylated. This includes protein needed for activation of checkpoints such as p53 and chk2 and repair of damaged DNA such as 53bp1 and brca1 (Matsuoka et al., 2007) (Ciccia & Elledge) (Jackson & Bartek, 2009) and (Bartek & Lukas, 2007) (Kennedy & D'Andrea, 2006). Moreover, activation of ATM allows the phosphorylation of C-terminus of H2AX, giving rise to phosphorylated H2AX ( $\gamma$ -H2AX).  $\gamma$ -H2AX allows binding of mdc1 proteins which will act as docking site for MRN-ATM complex (Lou et al.) Stuki et al. 2005; (Melandar et al., 2008)). As a result, ATM will phosphorylate more H2AX which will spread over several kilobases over the chromatin (Chatterjee, Fong, & Zhang, 2008) Whereas mdc1 protein will also recruit effector proteins such as RNF8 and RNF 168 ubiquitin ligases that will allow chromatin ubiquitination to facilitate the loading of 53bp1 and brca1 (Doil et al.2009).This will be followed by DNA repair through different pathways that will be discussed in the following section.



**Figure 2. Overview of chromatin remodeling.** Upon DSBs induction, different proteins will be directed to the damaged site to phosphorylate H2AX and facilitate the loading of 53bp1. Consequently, chromatin is relaxed and accessible for DNA repair.



#### 1.2.4 DNA Double strand breaks repair pathways.

There are two main mechanisms for DNA DSBs repair: Homology-dependent error free homologous recombination repair (HRR) and possibly error prone, classical DNA-PK-dependent non homologous end joining(c-NHEJ). These two pathways vary in their need of homologous template of DNA and in reliability of the DNA repair (Dexheimer, 2013). c-NHEJ is found to predominate in all of the cell cycle phases and can be classified into two different subways with two different repair kinetics: resection dependent c-NHEJ, slow repair pathway, and resection independent c-NHEJ, fast repair pathway. On the other side, HRR is restricted to late S-and G2-phases of the cell cycle and can be considered as more complex pathway with slower kinetics than c-NHEJ (Iliakis, Murmann, & Soni, 2015). It is now widely accepted that in case of inactivation of c-NHEJ repair or abrogated HR repair at G2-phase a backup pathway known as alt-EJ repair will take place at a cost of elevated chromosome translocation (Iliakis et al., 2015)

##### *Homologous recombination repair (HRR)*

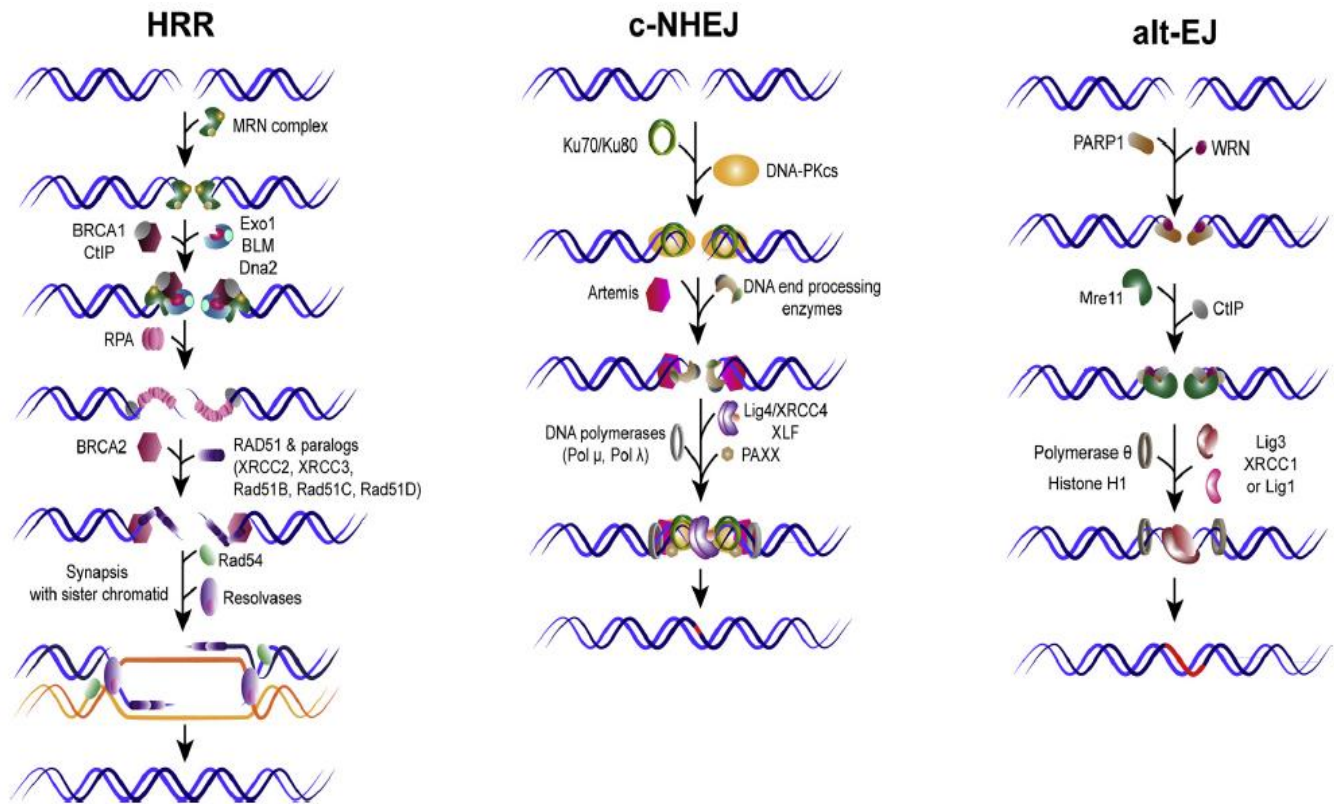
Homologous recombination repair can be divided into three different phases: presynapsis, synapsis and post synapsis (Dexheimer, 2013). In presynapsis phase the DNA at the DSBs are resected through 5'to3' ends to generate 3' single strand overhang. This process is highly regulated by the cell cycle and involves many proteins such as MRN, CtIP, EXO1, Dna2 and BLM helicase. The generated ssDNA is tightly coated with RPA, a replication protein, for stabilizing the secondary structures and preparing for RAD51 efficient filament formation by the help of Rad51 paralogs (Rad51b, Rad51C, Rad51D, Xrcc2, Xrcc3 and BRCA2). Next, during synapsis Rad51 efficient filament starts to search for homology which is the central reaction of HR. RAD51 promotes DNA strand invasion reaction by promoting the damaged DNA strand to invade the template DNA strand of the sister chromatids. Finally, post synapsis takes place by DNA synthesis starting from the 3' end of the invading strand by the help of DNA polymerase. This is followed by ligation using DNA ligase I to produce structure called "Holliday junction", four-way junction intermediate structure. This recombination intermediate structure is then released by three different ways either dissolution mediated by the BLM-TopIII complex, symmetrical cleavage using GEN1/Yen1 or Six1/SIX4, or by asymmetric cleavage using Mus81/Eme1 to produce error-free repair of the DSB (Dexheimer, 2013). This shows the molecular complexity of HRR and requirement for homology search and synapsis with homologous DNA strand. Thus, it is clear that repair by HRR is a slow repair pathway that would take time by the cell to achieve complete repair but once the repair is achieved it is considered to be error- free (Iliakis et al., 2015)

### *Classical DNA-PK-dependent non homologous end joining.*

During this pathway, removing DSBs from the genome is promoted by enzymatic rejoining of processed ends without the need for Homologous DNA template by fast kinetics (Iliakis et al. 2015). This is achieved by the capturing of broken DNA ends to Ku heterodimer (Ku70/80) which will allow recruitments of DNA-PKcs to the broken ends. The recruited DNA PKcs will show conformational changes at the site of DSB to dimerise in order to produce a scaffold for consequent processing of the DNA broken ends. This is followed by ligation of compatible ends by ligase IV/XRCC4 complex (Ochi et al., 2015), (Xing et al., 2015). However, in many cases DNA ends are not compatible and needs further processing before ligation. This is done by DNA polymerases, polynucleotide kinase and Artemis nuclease for processing non compatible ends for subsequent DNA polymerization. In some cases, DSBs induction by PI3K initiates resection-dependent c-NHEJ that will repair the DSBs by slower kinetics (Iliakis et al., 2015). During resection-dependent c-NHEJ repair PI3K phosphorylates CtIP at ser327 to allow CTIP-Brc1 interaction. Following initiation of CTIP-Brc1 interaction, Brc1 relieves 53Bp1 barrier to resection. This relief allows Mre11 exonuclease, EXD2 and Exo1 execute resection for Artemis to finish the process. Although c-NHEJ pathway has the advantage of repairing the DSBs faster than HRR, it has the limitation of introducing error prone repair due to absence of homologous DNA template (Iliakis et al., 2015)

### *Alternative-end joining repair pathway (alt-EJ)*

Some studies showed that alt-EJ reflects several dedicated pathways to DSBs repair due to detection of occasional preferences of some proteins from processing of DSBs (Oh et al 2014. (Simsek et al., 2011). Other showed alternative view, alt-EJ is not functionally specific DSB repair pathway, but it is “a backup” repair pathway that the cell utilizes to remove any unprotected DNA ends in existence of pre-enzymatic activities (Iliakis et al., 2015). Thus, whenever the main pathway either c-NHEJ or HRR, fails to repair the DSBs, alt-EJ will take place as a backup to protect the damaged DNA at any price. This alternative view contradicted the first view by reflecting that the detection of some proteins can show at which stage c-NHEJ or HRR failed rather than showing a pre-designed specific pathway of alt-EJ. alt-EJ backup pathway is known to be more error prone due to slower kinetics of this pathway which allows more possibility of chromosomal translocation than c-NHEJ repair pathway (Iliakis et al. 2015).

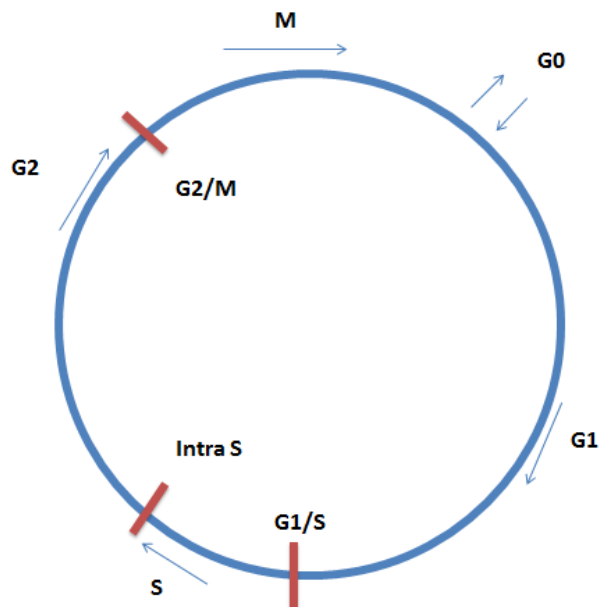


**Figure 3. Overview of DNA DSBs repair pathways. HRR, Homologous recombination repair, c-NHEJ, classical- non homologous end joining, alt-EJ, alternative end joining. (Iliakis et al., 2015).**

## **1.3 Cell cycle phases and Cell checkpoints**

### **1.3.1 Cell cycle phases**

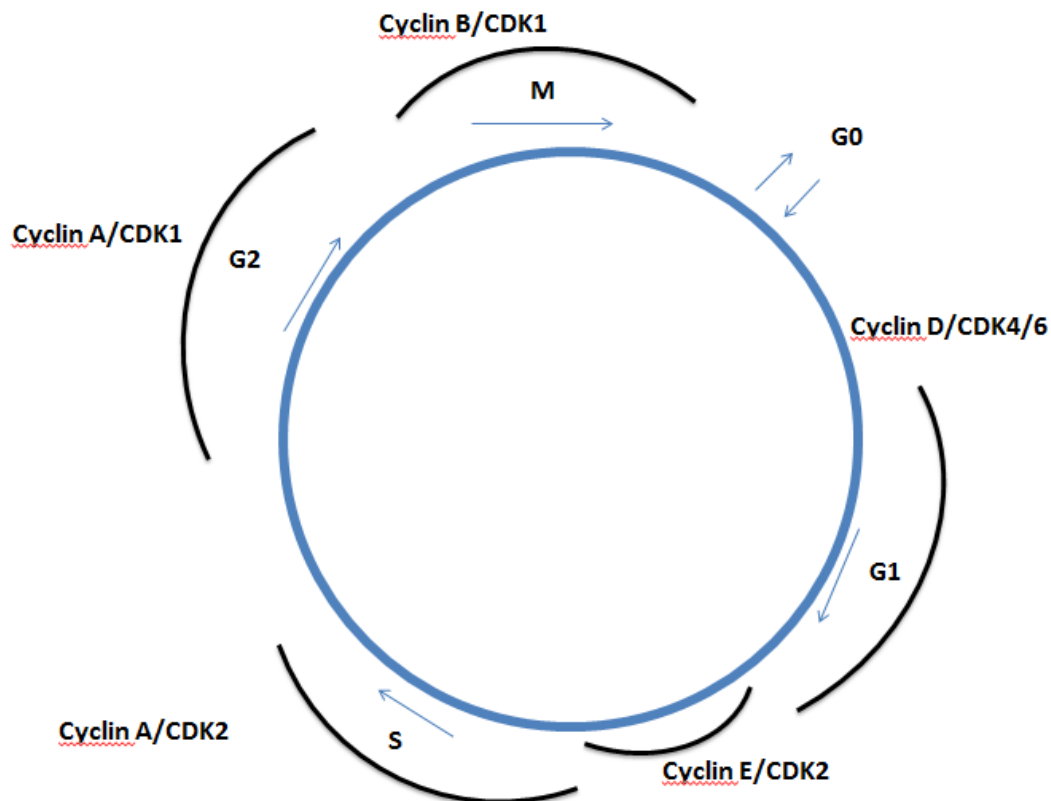
The Cell cycle is regulated events where cellular components grow, DNA accurately doubled and divided into two cells (Hartwell & Weinert, 1989). These events are achieved in a cycle rather than linear pathway because at the end of each cycle daughter cells can re-start the exact process from the beginning. The mammalian cell cycle is composed of two main phases: Interphase and Mitosis. The Interphase, cell grows and makes a copy of its DNA to prepare for mitosis. Then during Mitosis, the two copies of DNA are separated into two sets and the cytoplasm is divided to give two daughter cells. Interphase consists of three different phase : G1-S-G2 phase. G1 phase, a phase in which the cell starts to prepare for DNA replication by increasing in size and beginning RNA and Protein synthesis. Then S-phase in which DNA semi-conservative replication happens giving two identical copies of the genetic information carried by DNA, and duplication of the centrosome, which will help in DNA separation during Mitosis. Then comes G2 phase in which cells grows more and the RNA and Protein synthesis initiated in G1 is completed to allow the cell to enter mitosis. During mitosis cell divides its two DNA copies and cytoplasm to give two identical daughter cells through two-distinct division-related processes: mitosis and cytokinesis. Finally, daughter cell exits the cell cycle in two different ways according to the type of cell. If it is rapidly dividing cell then cell will re-start immediately another round of cell division, if it is slowly dividing cell then the cell will exit G1 phase and enter resting state called G0 phase. G0 is sometimes permanent state for some cells while other cells can exit G0 and re-divide upon receiving specific signals (Hartwell & Weinert, 1989).



**Figure 4. Cell cycle phases and cell cycle checkpoints. (Modified from Houtgraaf et al. 2006)**

### 1.3.2 Cell cycle checkpoints

To ensure accurate propagation of DNA copies within generations, cell responds to DNA damage or abnormally structured DNA by arresting the cell cycle at different cell cycle checkpoints. Cell cycle checkpoint is inspection mechanism that examines the reliability and the fidelity of major events in the cell cycle. This is achieved by network of signaling pathways arresting the cell to allow enough time for DNA repair of the damaged sites, or allow cell senescence if the damage is beyond repair. These biochemical signaling pathways includes sensor proteins which will search for any damage in the genome, and initiate different signaling pathways upon DNA damage to be amplified or propagated by adaptor/ mediator proteins and signal transducers to check point effectors to delay progression or transition through major cell cycle events until DNA damage is repaired. Thus, the movement through cell cycle phases is tightly maintained by expression of checkpoint protein complexes that will allow biochemical signaling pathways for cell cycle arrest. These complexes are known as cyclin/Cdk complexes that are consists of two subunits: a regulatory subunit known as cyclin and a protein kinase known as cyclin dependent kinase (Cdk). Expression of different Cyclin/Cdk complexes depends on the phase of the cell cycle (Lukas, Lukas, & Bartek, 2004). The following diagram shows an overview for the regulation of the checkpoints.



**Figure 5. Cell cycle regulation by cyclin-dependent kinase. Modified from (Suryadinata, Sadowski, & Sarcevic, 2010)**

### *G1 checkpoint*

DNA damage to cells in G1 phase will activate cell cycle checkpoint transducing kinases : ATM and ATR. This is followed by targeting the effector protein Cdc25A and the transcription factor p53 via Chk1/Chk2. The activation of two effector proteins leads to two different pathways. In the first pathway, DNA damage will activate Chk1/Chk2 to phosphorylate Cdc25A, which will leads to ubiquitination and protein degradation. This is followed by inhibition of CDK2 consequently leads to inhibition of the entry to S-phase. This pathway is known to be rapid activation and halt the progression of the cell cycle 1-2 hours (Lukas et al., 2004). During the second pathway, same transducers Kinases (ATM and ATR) will be activated but this time they will target p53 and p21 effector proteins. Following DNA damage ATM/ATR phosphorylates p53 via Chk1/Chk2. Also ATM/ATR targets p53's negative regulator, Mdm2. Thus, accumulation of p53 will occur which will act as transcription factor for CDK inhibitor p21. After several hours the accumulation of p21 will prevent the cell cycle progression into S-phase. Thus, this pathway seems to sustain or sometimes permanent cell cycle blockade(Lukas et al., 2004)

### *S-Checkpoint.*

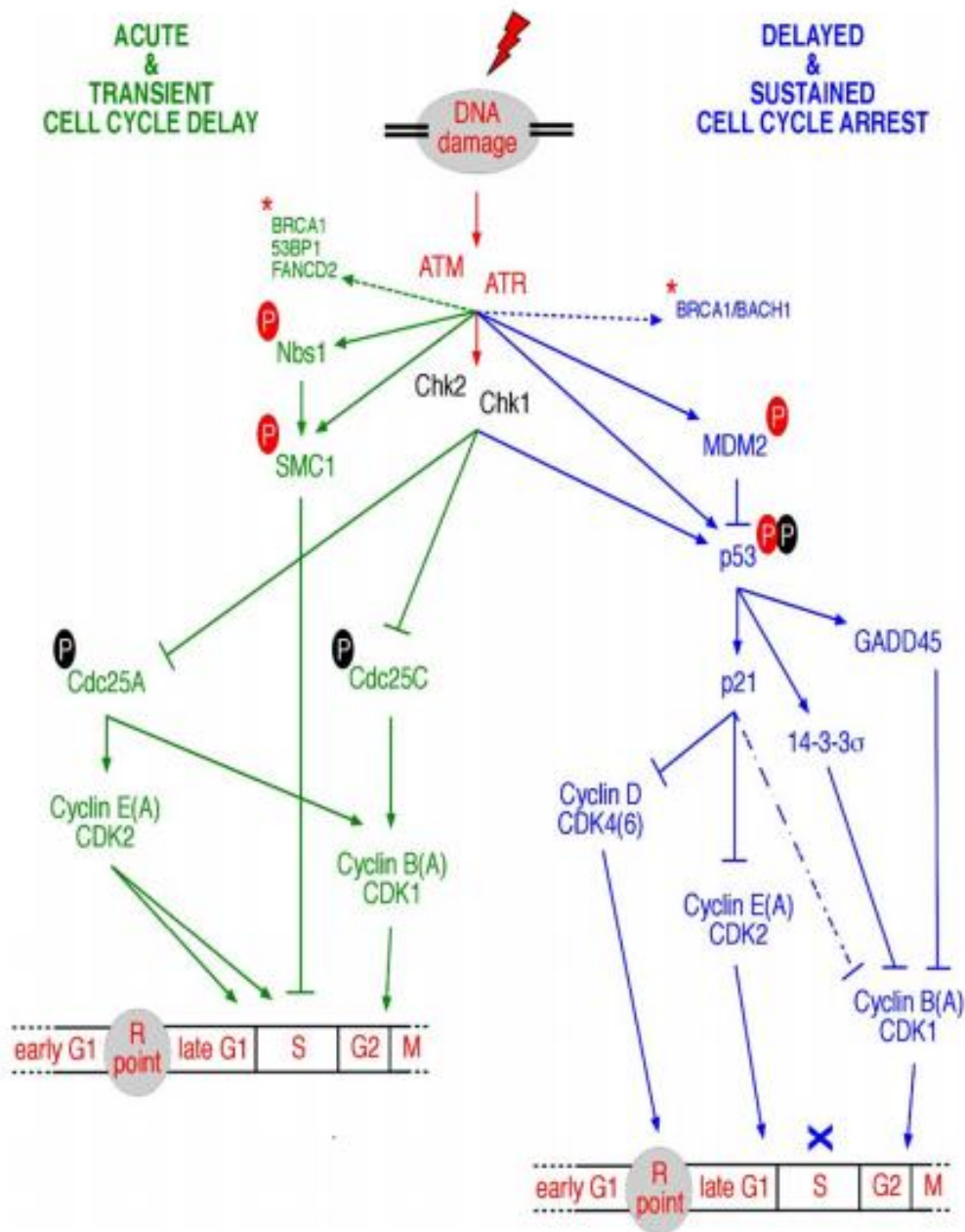
Unlike G1 and G2/M checkpoints, activation of S-phase cell cycle checkpoint is independent of p53 and causes reversible delay of the cell cycle progression by slowing down DNA replication (Bartek & Lukas, 2001) Upon exposure to genotoxic agent during S-phase the S-phase checkpoint is activated through different pathways. The first pathway is ATM/ATR-Chk2/Chk1-Cdc25A-CDK2 pathway which causes delay in the cell cycle in G1 phase. The second pathway, ATM phosphorylates of NBS1, a part of Mre11/Rad 50/ NBS1 complex. Thus, NBS1 is directed towards the damaged site for processing DNA broken ends. Also,SMC1 is considered to be another substrate for ATM that upon it's phosphorylation at serines 957 and 966 will cause DNA synthesis inhibition (Shackelford, Kaufmann, & Paules, 1999)

### *G2/M checkpoint.*

The G2/M checkpoint inhibits cells to enter mitosis when the cell faces DNA damage in G2 phase. Thus, cell cycle will be arrested to prevent entry of mitosis with damaged DNA. Similarly to G1 checkpoint, and unlike S-phase checkpoint, G2 checkpoint has two different pathways which will leads to acute or sustained checkpoint activation. Both pathways lead to inhibition of Mitosis through inhibition of B/Cdk1 kinase. The first pathway is an acute pathway that will leads to acute cell cycle delay. This is achieved by ATM/ATR-Chk1/Chk2 inhibition to B/Cdk1 kinase via cellular sequestration and inhibition of Cdc25 phosphatases which will eventually leads to inhibition of mitosis. The second pathway which will results in sustained arrest that will be by activated via Cdk inhibitor p21. Cdk inhibitor p21 expression is regulated by p53. GADD45 and 14-3-3 $\sigma$  are more transcriptional goals of p53. Thus, p21, GADD45 and 14-3-3 $\sigma$  leads to inhibition of B/Cdk1 that will inhibit mitosis and allow sustained cell cycle arrest (Shackelford et al., 1999). There is evidence that



G2/M checkpoint is partly independent of p53. This is because it was shown that the loading of BRCA1 induces p21 and GADD45 in sustained G2 arrest pathway (Li et al., 2000)



**Figure 6. Overview of the molecular pathways leading to G1, S and G2/M arrest (Lukas et al., 2004)**

### **1.3.3 The master G1 and The negligent G2/M checkpoints.**

Recent studies showed that G2/M cell cycle checkpoint activation has threshold of 10-20 DSBs. This reflects insensitivity of G2/M checkpoint to low amount of DNA damage which will leads to proliferation of cells with damaged DNA. As a result, low level of endogenous and exogenous DNA damage results in carcinogenesis. For instance, exposure to low concentration of TiO<sub>2</sub> NPs will induce low amount of DNA damage that will not activate G2/M checkpoint and cells will proliferate in presence of DNA damage. Whereas exposure to high concentrations of TiO<sub>2</sub> NPs will activate G2/M checkpoint for DNA repair. However on repeated exposure to high concentrations of titanium dioxide NPs cell will not have enough time to fully repair the DNA damage so it will maintain significant amount of unrepaired DNA damage. Therefore, the negligent G2/M checkpoint will not maintain cell cycle arrest for long time and will lead to cellular adaptation allowing the cells to divide in presence of DNA damage induced by NPs leading to genetic instability.

However, there are several results that proposed that G1 checkpoint has higher sensitivity to single DNA damage than G2/M checkpoint (Löbrich & Jeggo, 2007). This is because it was shown that after release of cell cycle checkpoint G2/M cell with unrepaired DNA damage shows decrease in cell survival. This shows that the cells could be eliminated from the cell cycle by more sensitive checkpoint as G1 cell cycle checkpoint. Also it was shown that single DSB after microinjection with linearized plasmid can initiate G1 cell cycle checkpoint (Huang, Clarkin, & Wahl, 1996) Lastly, it was shown that shorten telomeres arrest the cell cycle in G1 phase although it is known that shortening of telomeres arise in replication of DNA and needs progression of G2/M checkpoint phase to enter G1 phase (Fagagna et al., 2003)



## **2. Experimental design and Methods.**

### **2.1 Cell lines**

The study consists of two parts. The first part is evaluating the changes in cytotoxicity, genotoxicity and cell cycle checkpoint maintenance on nontransformed and transformed cell lines after acute and prolonged exposure to different concentrations of TiO<sub>2</sub> NPs. Consequently, 1 br hTERT, immortalized normal human fibroblasts, U2OS, Human Bone Osteosarcoma epithelial cells, and A549, Adenocarcinomic human alveolar basal epithelial cells, were used.

The second part is identifying the type of DNA damage induced by NPs, the possible mechanisms of DNA repair and the proteins needed to repair DNA damage induced by NPs and the proteins needed in regulating G2/M cell cycle checkpoint. In this part cell lines will be investigated after acute exposure to low concentration of TiO<sub>2</sub> NPs. These different cell lines include wild type cell line and other cell lines that have different proteins needed in controlling cell cycle checkpoints and repairing damaged DNA knocked out. For instance, WT, mouse embryo fibroblasts, ATM<sup>-/-</sup>, ATM mutated mouse embryo fibroblasts, ART<sup>-/-</sup>, Artemis defective mouse embryo fibroblasts, and DNA Pkcs<sup>-/-</sup>, DNA-Pkcs defective mouse embryo fibroblasts, cell lines will be used.

## **2.2 Aim of the study.**

The aim of this study is to investigate the impact of exposure to low concentration of TiO<sub>2</sub> NPs on induction of DNA damage, cell cycle checkpoints and DNA repair. The experiments will be carried out on normal cell lines: WT, mouse embryo fibroblasts, and 1Br hTERT, immortalized normal human fibroblasts, cancer cell lines: A549, Adenocarcinomic human alveolar basal epithelial cells, and U2OS, Human Bone Osteosarcoma epithelial cells as well as DNA repair deficient cell lines: ATM, ATM mutated mouse embryo fibroblasts,, and ART, Artemis defective mouse embryo fibroblasts, DNA PKcs defective mouse embryo fibroblasts

Using these Cell lines, the type of DNA damage induced by NPs will be investigated. This will be followed by comparing the amount of DNA damage following acute and prolonged exposure to NPs in normal and cancer cells. Also, the impact of NPs' acute and prolonged exposure on activating and maintaining cell cycle G2/M checkpoint in normal, cancer as well as cell cycle checkpoint deficient cells will be investigated. Finally, DNA repair pathways expressed upon induction of DNA damage by NPs on normal and DNA repair deficient cells will be investigated.

**Hypothesis I. Acute and prolonged exposure to TiO<sub>2</sub> NPs induces toxicity in normal as well as cancer cell lines.**

**Hypothesis II. ATM and DNA PKCs have role in DNA repair of DSBs induced by the NPs.**

**Hypothesis III. ATM has role in activating G2/M checkpoint after induction of DSBs by the NPs.**

## **2.3 Materials and Methods**

### **2.3.1 Chemicals and Nanopowders**

All chemicals, cell culture media and supplements were purchased from Sigma Aldrich. Titanium (IV) oxide, nanopowder, 21nm primary particle size (TEM), >99.5% trace metal basis was obtained from Sigma Aldrich (ref. 718467)

### **2.3.2 Nanoparticle dispersion**

Powdered TiO<sub>2</sub> NPs were suspended in fresh growth media, Dulbecco's Modified Eagle Medium, DMEM, at a concentration 200 µg/ml. Just before exposure, the NPs are sonicated for 30 minutes using High power probe sonication (QSonica, Q700, Sonicator) in pulsed mode (1s on/1s off), at 4°C and 28% of amplitude. Then Suspensions were diluted in growth media, DMEM, to yield concentrations ranging from 0.1–200 µg/ml.

### **2.3.3 Cell culture**

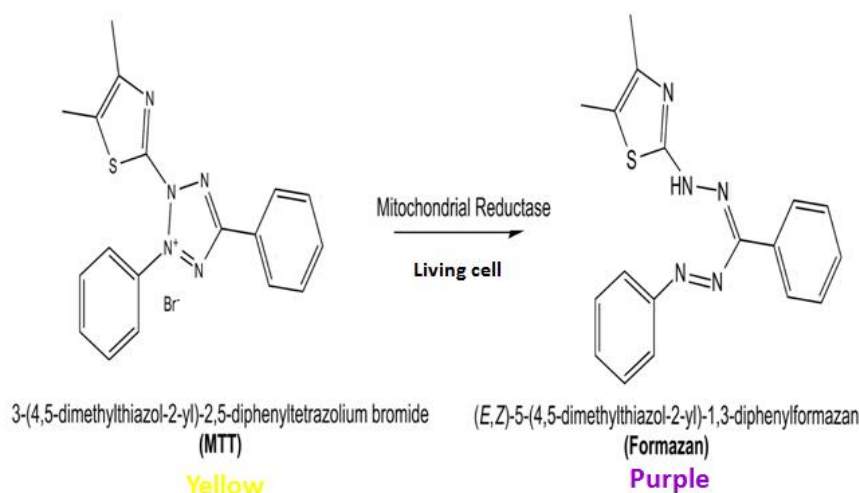
A total of seven cell lines are to be used in this study. These are:

1. 1 BrhTERT, immortalized normal human fibroblasts (Control).  
(Purchased from ATCC)
2. WT, Wild type mouse embryo fibroblasts (Control).  
(Generous gift from Jeggo lab, Genome Damage and Stability Centre, UK)
3. U2OS, Human Bone Osteosarcoma epithelial cells.  
(Purchased from ATCC)
4. A549, Adenocarcinomic human alveolar basal epithelial cells.  
(Purchased from ATCC)
5. ATM<sup>-/-</sup>, ATM mutated mouse embryo fibroblasts.  
(Generous gift from Jeggo lab, Genome Damage and Stability Centre, UK)
6. ART<sup>-/-</sup>, Artemis defective mouse embryo fibroblasts.  
(Generous gift from Jeggo lab, Genome Damage and Stability Centre, UK)
7. DNA Pkcs<sup>-/-</sup>, DNA-Pkcs defective mouse embryo fibroblasts.  
(Generous gift from Jeggo lab, Genome Damage and Stability Centre, UK)

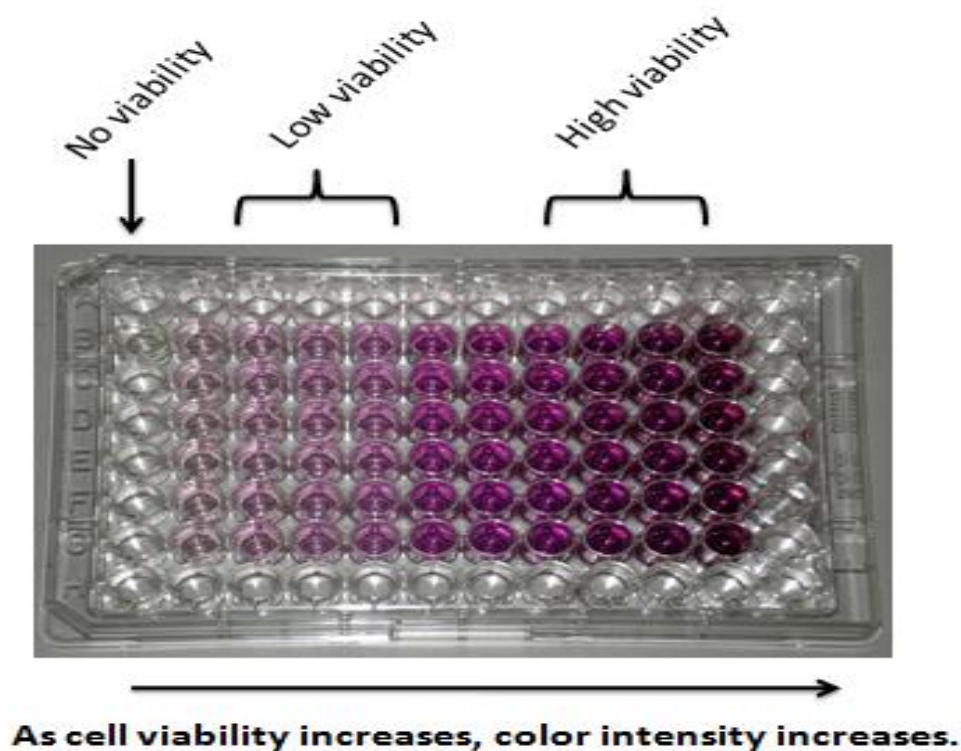
All cell lines were subcultured in fresh growth media, DMEM containing 4.5 g.l<sup>-1</sup> glucose supplemented with 2 mM L-glutaminpenicillin/streptomycin (50 IU.mL<sup>-1</sup> and 50 mg.ml<sup>-1</sup>, respectively) and 10% (vol/vol) fetal bovine serum (FBS). The cells were Kept at 37 °C in a humidified 95% and 5% CO<sub>2</sub> air. The cells were passed when they reach 80% confluency in T75 flask.

### 2.3.4 MTT

It is a Sensitive colorimetric assay for assessing cell viability. Mitochondrial NAD(P)H-dependent cellular reductase enzymes can show the number of living cells. This is because these enzymes can be used to reduce yellow soluble tetrazolium dye, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, to purple insoluble formazan, which can be dissolved in solubilizing solution such as Dimethyl sulfoxide, DMSO, giving purple colored solution (Mosmann, 1983). Absorbance of the colored solution can be measured at wavelength 530 nm using spectrophotometer. This shows that the reduction of MTT into colored formazan product will occur only in metabolically active cells. Thus, the more viable cells present the greater reduction of yellow tetrazolium dye into purple formazan giving darker purple color, which will result in higher absorbance by spectrophotometer.



**Figure 7. MTT reduction by living cells (Ebada, Edrada, Lin, & Proksch, 2008).**



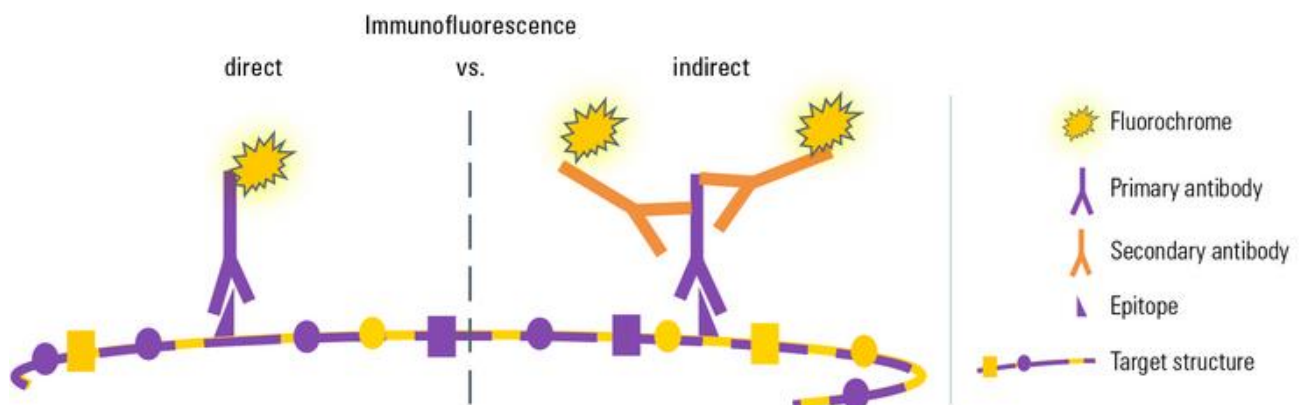
**Figure 8 . Micro titer plate after MTT assay. Increasing the amount of viable cells result in increased purple coloring.**

Cells were seeded and grown to reach confluence in 96-well plates before being exposed to 100  $\mu\text{L}$  of 0.1–200  $\mu\text{g}/\text{ml}$  of NPs suspension for 24–168 hours. After NPs' exposure, 100  $\mu\text{L}$  of a 5 mg/ml MTT solution were added to each well. After 4 hours incubation at 37  $^{\circ}\text{C}$  in dark, medium was then replaced by 100  $\mu\text{L}$  of DMSO, dimethyl sulfoxide, and mixed for 1 minute on plate reader's shaker until the formazan crystals are dissolved. In order to avoid the effect of the remaining of the NPs present within the wells that may affect the absorbance, NPs were allowed to settle after dissolving the formazan crystal and 50  $\mu\text{l}$  were transformed from each well to new plate. Then, absorbance was measured at 570 nm wavelength and cell survival was calculated as a percentage of the negative control (unexposed cells).

### 2.3.5 Immunofluorescent staining

It is a technique used by fluorescence microscope to detect the presence of specific proteins. There are two ways to detect the presence of specific proteins using immunofluorescent staining: primary (direct) or secondary (indirect) immunofluorescent assay.

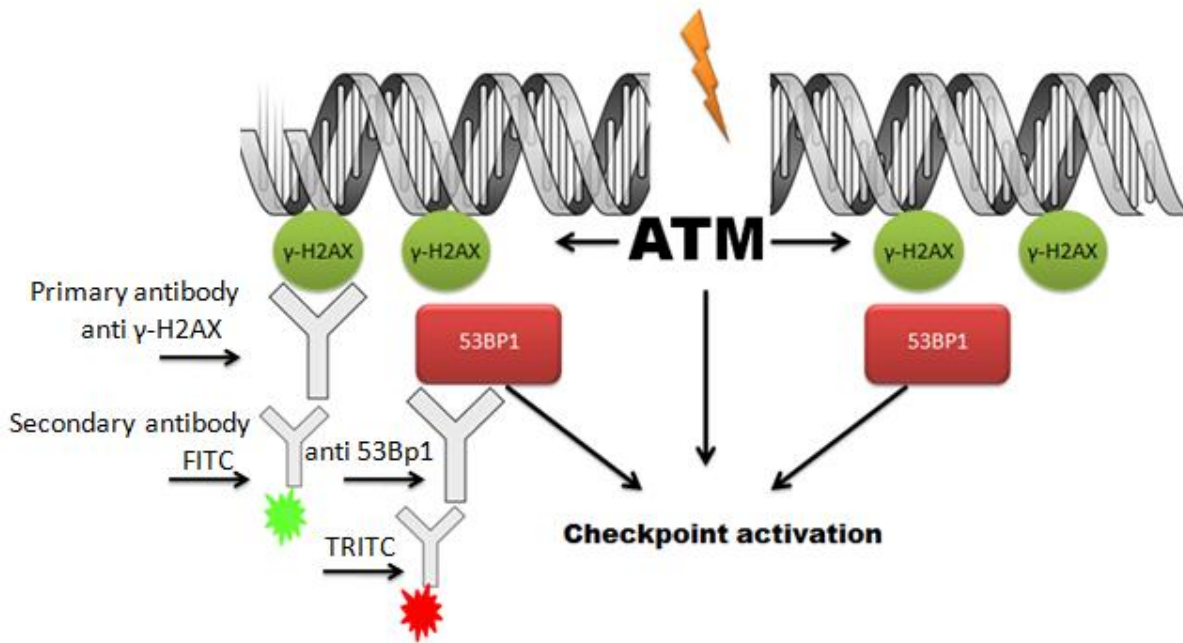
Primary (direct) Immunofluorescence utilizes a single primary antibody which is conjugated to a fluorescence substance. Primary antibody will binds to the target molecule and the attached fluorophore could be identified using fluorescent microscopy. While secondary (indirect) Immunofluorescence utilities two, primary and secondary, antibodies. Unlabeled primary antibody used to bind to the target by its variable region and multiple secondary labeled antibodies could target single primary antibody at its constant region. Thus, amplification of the signal will occur and increases the fluorophore molecules per target. Although, the direct immunofluorescence has less number of steps in the procedure, it is less sensitive than the indirect immunofluorescence and may give false negative results. Thus, we decided to use indirect immunofluorescence to amplify the signal used to detect our targets in this project.



**Figure 9. Overview showing the difference between primary Immunofluorescence and Secondary Immunofluorescence techniques (Hoff et al, 2015).**

### 2.3.6 $\gamma$ -H2AX and total 53BP1 immunofluorescent staining

In this assay immunofluorescent staining for detection of DNA double strand breaks using anti- $\gamma$ H2AX and anti-total-53BP1 as a primary antibodies will be performed. This is because upon induction of DSBs, sensor proteins MRE11-RAD50-NBS1, MRN complex, localizes ATM to the site of DSB. Simultaneously, auto phosphorylation of DNA-PKcs proceeds. Together, ATM and DNA-PKcs phosphorylates H2AX at serine 139 forming  $\gamma$  H2AX this serves as platform for recruitments of different proteins that will leads to recruitment of 53BP1 and activation of checkpoint for DNA repair functions (Figure 10). Thus, quantifying both  $\gamma$ -H2AX and 53BP1 foci will serve as distinctive biomarkers for quantifying the amount of double strand breaks per cell (Watters, Smart, Harvey, & Austin, 2009). As a result anti  $\gamma$ H2AX and anti-total-53BP1 will be used as primary antibodies that recognizes the phosphorylated  $\gamma$ H2AX and 53BP1 while secondary antibodies labeled with FITC and TRITC will binds to anti-  $\gamma$ H2AX and anti-total-53BP1 respectively, producing fluorescence that will be visualized using fluorescent microscope.



**Figure 10. Overview showing the visualization of  $\gamma$ -H2AX and 53BP1 proteins using secondary (indirect immunofluorescence).** Primary antibodies for  $\gamma$ -H2AX and 53BP1 were used as distinctive biomarkers for identification of DSBs. The visualization of primary antibodies were carried out using labeled secondary antibodies. One of the secondary antibodies was labeled with FITC to give green foci in presence of  $\gamma$ -H2AX and other one was labeled with TRITC to give red foci in presence of 53BP1.

Cells were seeded on cover slips using 3 cm Petridish and exposed to NPs. After NPs exposure cells were fixed for 10 minutes in 3% formaldehyde, and permeabilized using Triton X-100 in PBS for 3 minutes. This was followed by adding 100 $\mu$ l of anti- $\gamma$ -H2AX and anti-53BP1 as primary antibodies (dilution 1/600, vol./vol in 2% BSA) for 1 hour at room temperature. Then cells are washed three times with PBS and 100  $\mu$ l of Secondary antibodies labeled with FITC and TRITC (dilution 1/200, vol./vol. in 2% BSA) were added at room temperature for 1 hour in complete darkness. Cells nuclei were stained for 5 minutes with DAPI (0.005 mg/ml) in complete darkness. Cover slips were mounted on slides using fluorescein media for microscope analysis. Average numbers of green Foci that co-localized with red foci were measured per 50–100 cell nuclei, using inverted microscope Olympus TM using WU and WB filters at wavelength ranges 358-461 and 495-570nm respectively. Three slides were analyzed in each condition.



### **2.3.7 PhosphoHistone H3 Immunofluorescent staining**

As mentioned above, the G2/M checkpoint do not allow G2-phase cells have DSBs to enter M-phase. The G2/M checkpoint assay assesses the progression of exposed cells from G2 into mitosis. Phosphorylation at Ser10 of histones H3 is associated with chromosome condensation during mitosis (Goto et al., 1999). (Hendzel et al., 1997); thus, primary antibody that precisely recognizes the phosphorylated form of histone H3 (p-histone H3 Ser10), and secondary antibody labeled with TRITC that will produce fluorescence upon binding to primary antibody are used to identify mitotic cells under fluorescent microscope.

Cells were seeded in 12 wells plate and left to attach over night. When cells reached exponential phase NPs were added. After exposure, cells were fixed in 3% formaldehyde for 10 minutes. After fixation, cells were washed with PBS three times. Then cells are permeabilized with 0.1% Triton X-100 for 3 minutes and washed again with PBS three times. Then 100 $\mu$ l of primary antibody for phosphorylated-H3 was added for 1 hour at concentration 1:300 in 2% BSA. Then 100 $\mu$ l of secondary antibody, TRITC, was added for 1 hour at concentration 1:100 in 2% BSA in complete darkness. Cell nuclei were stained with DAPI (0.005mg/ml) for 5 minutes. Cells were visualized using inverted microscope Olympus TM using WU and WB filters at wavelength ranges 358-461 and 495-570nm respectively. Three plates were analyzed in each condition.

### **2.3.8 Statistical Methods**

Each experiment was performed three times and mean values were calculated. The significance of the mean values for different conditions was assessed by analysis of variance, ANOVA, for single and two factors. The difference was considered significant when p value is less than 0.05. Error bars are expressed as percentage of Confidence intervals.

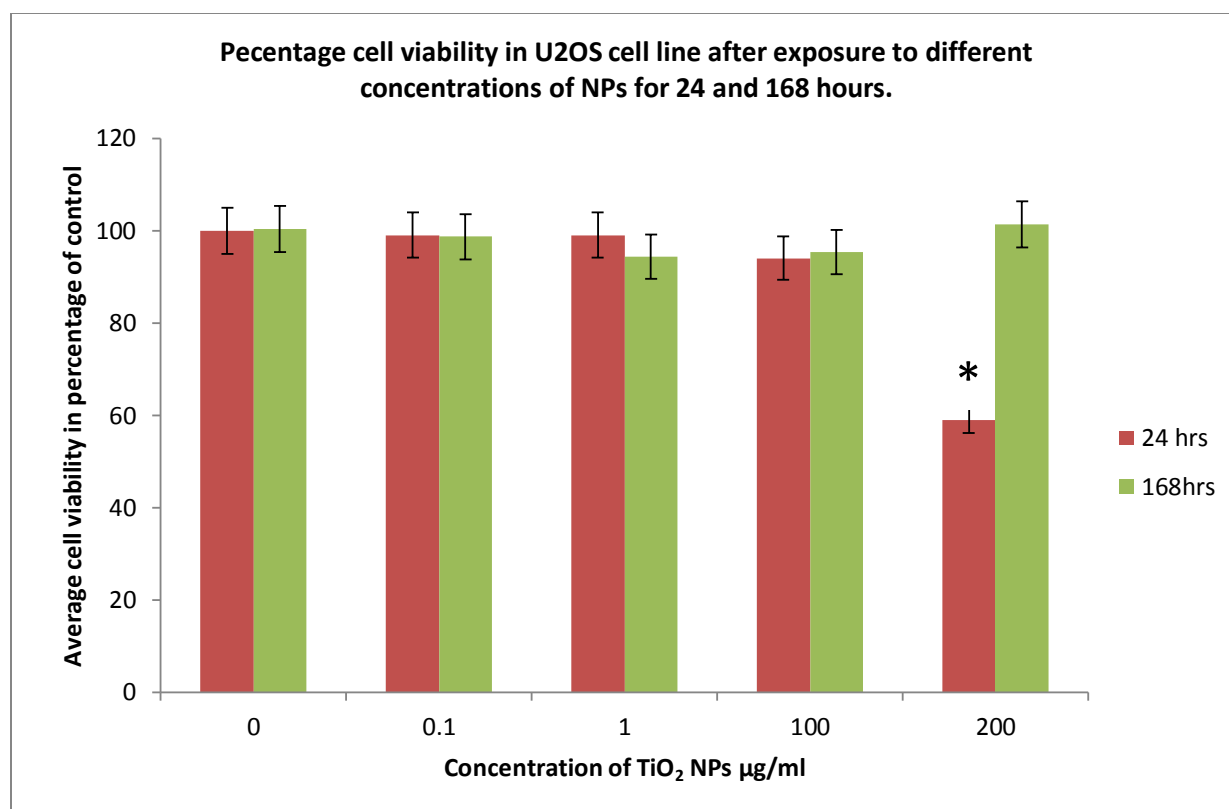
### **3. Results**

#### **3.1 Characterization of the Nanoparticles.**

In this study we did not characterize the NPs we used. However, the same NPs were characterized by another study (Franchi et al., 2015) through energy dispersive X-ray spectrometry, EDX. It was found that the NPs are relatively pure without contaminants. Also using Scanning electron microscope, SEM, it was found that NPs are spherical in shape and in range between 28 and 49 nm in size. Before cellular exposure, NPs were suspended in Complete cell medium containing 10 % FBS by sonication (Franchi et al., 2015). Thus, Hydrodynamic size and surface potential of the NPs were assessed in 10 % FBS-containing medium after sonication and incubation for 24 hours by Dynamic Light Scattering, DLS using Malvern Zetasizer, Nano-ZS equipped with 4.0mW, 633 nm laser. It was shown that the hydrodynamic size of the NPs was much higher than their sizes in solid state. This suggests the formation of NPs agglomerates with serum proteins. Also, after incubation the NPs agglomerates increased by 9% from initial agglomerates size (Franchi et al., 2015). Finally, Zeta potential values for NPs were negative showing possible occurrence of NP-protein complexes (Franchi et al., 2015).

#### **3.2 Cytotoxicity**

Testing the survival and death rate after acute and prolonged exposure of the NPs gives a vision into the short as well as long term effect of the NP's ability to kill the cells. Using MTT assay, cytotoxicity of TiO<sub>2</sub> NPs was assessed in Human Bone osteosarcoma Epithelial cells (U2OS). After exposing the cells to different concentrations of TiO<sub>2</sub> NPs from 0.1 to 200 µg/ml for different exposure times 24 and 168 hours, the percentage of cell viability was significantly reduced from 100% to 53% after 24 hours at the highest concentration used. However, no significant decrease in percentage of cell viability was observed after 168 hours. Although there was no cytotoxicity observed after 168 hours we thought that may be genotoxicity induced and stabilized by the nanoparticles on prolonged exposure which leads to cellular adaptation rather than cellular death.

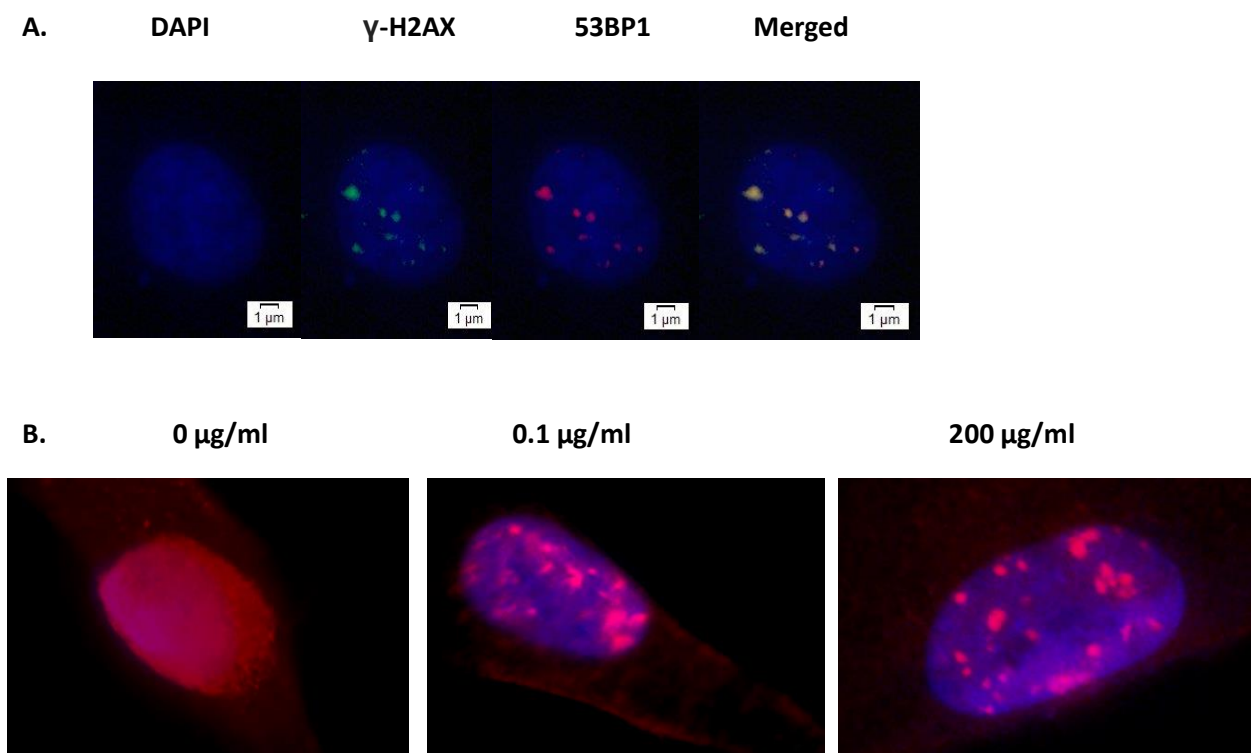


**Figure 11. Evaluation of cytotoxicity induced by TiO<sub>2</sub> NPs in U2OS cell line.** A significant concentration dependent increase in cytotoxicity in U2OS cells exposed for 24 hours ( $P < 0.05$ ). However, no significant cytotoxicity was observed after 168 hours ( $P > 0.05$ ). The data are expressed as mean values from three independent experiments, with  $n=6$  in each independent experiment.

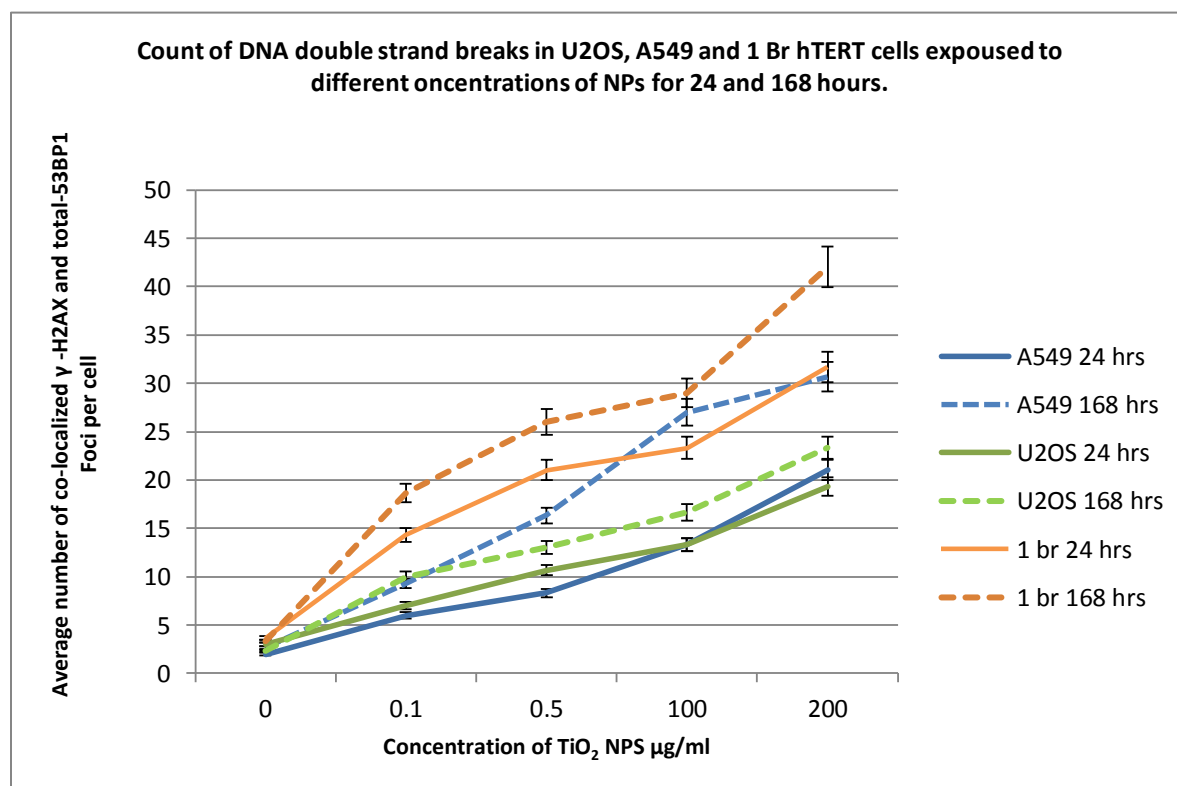
### **3.3 Genotoxicity**

#### **3.3.1 Immunofluorescent detection of DNA double strand breaks in U2OS, A549 and 1Br hTERT cell lines.**

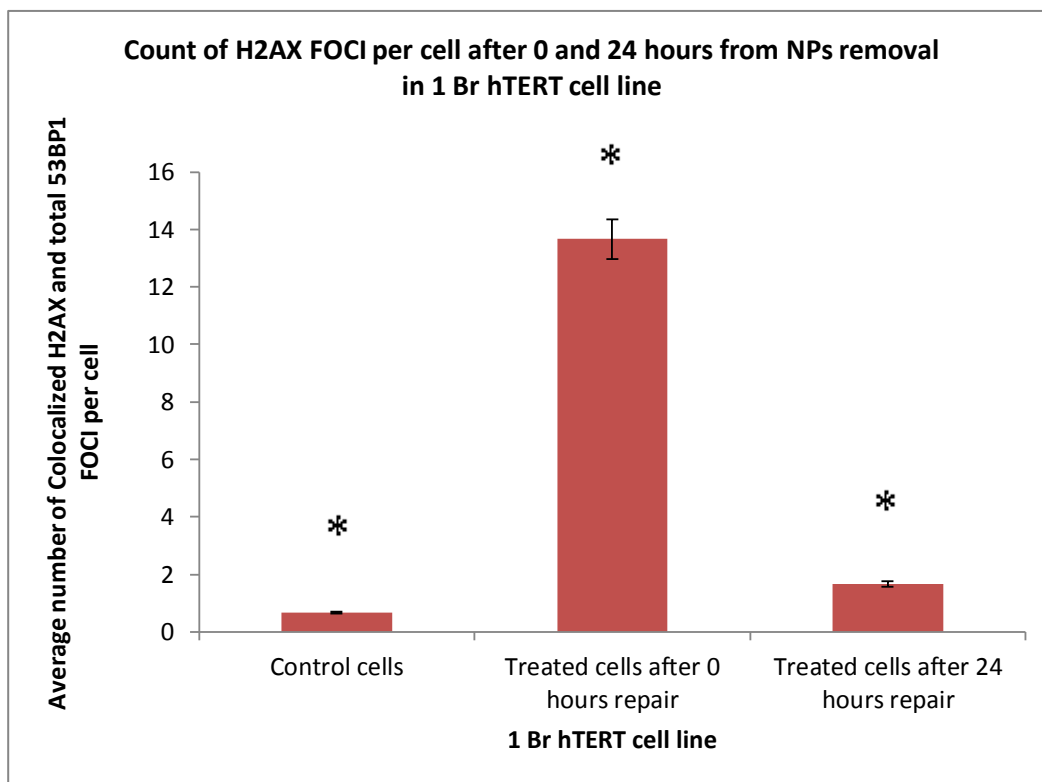
Cytotoxicity is not the only biological marker that could reflect the toxicity of the NPs. This is because the NPs may induce genotoxicity without affecting cell survival leading to mutation rather than cellular death. Therefore, we evaluated the DNA damage induced by the NPs and to compare the amount of DNA damage induced over acute and prolonged exposure. Sub-confluent U2OS, A549 and 1Br hTERT cells were exposed to different concentrations of TiO<sub>2</sub> NPs (0 - 0.1 - 0.5 - 100 - 200 µg/ml) for 24 and 168 hours then immunofluorescent staining for γ-H2AX and total-53Bp1 was performed immediately. As a result, significant dose- dependent genotoxicity was observed in the three cell lines. However no time-dependent genotoxicity was observed. This indicates that DNA repair may have occurred in between repeated exposures. Consequently, cells do not accumulate DNA damage over 168 hours showing no time dependent genotoxicity.



**Figure 12. Immunofluorescent Visualization of DNA double strand breaks in U2OS, A549 and 1Br hTERT cell lines.** **A.** Representative 2-D images of A549 cells Nuclei were stained with DAPI (Blue Nuclei) and cells were stained for phosphorylated H2AX ( $\gamma$ H2AX) (green Foci) and total-53BP1 (red Foci).  $\text{TiO}_2$  NPs induces  $\gamma$ -H2AX foci and 53BP1 that forms distinct foci which co-localized with  $\gamma$ -H2AX in the DAPI stained nucleus showing orange coloration. **B.** Representative 2-D images of dose-dependent 53BP1 foci formation in 1 Br hTERT cells after treatment with different concentrations of  $\text{TiO}_2$  NPs.



**Figure 13. Immunofluorescent quantification of DNA double strand breaks in U2OS, A549 and 1 Br hTERT cell lines.** Representative graph showing significant dose-dependent response curves for A549, U2OS and 1Br hTERT cell lines ( $p < 0.05$ ) and insignificant time-dependent response curves for A549, U2OS and 1 Br hTERT cell lines. DNA Double-strand breaks quantified by counting the average number of co-localized  $\gamma$ -H2AX and total-53BP1 Foci per cell in U2OS, A549 and 1Br hTERT cell lines treated with  $\text{TiO}_2$  NPs at different concentrations (0-200  $\mu\text{g/ml}$ ) for 24 and 168 hours. The data are expressed as mean values from three independent experiments.

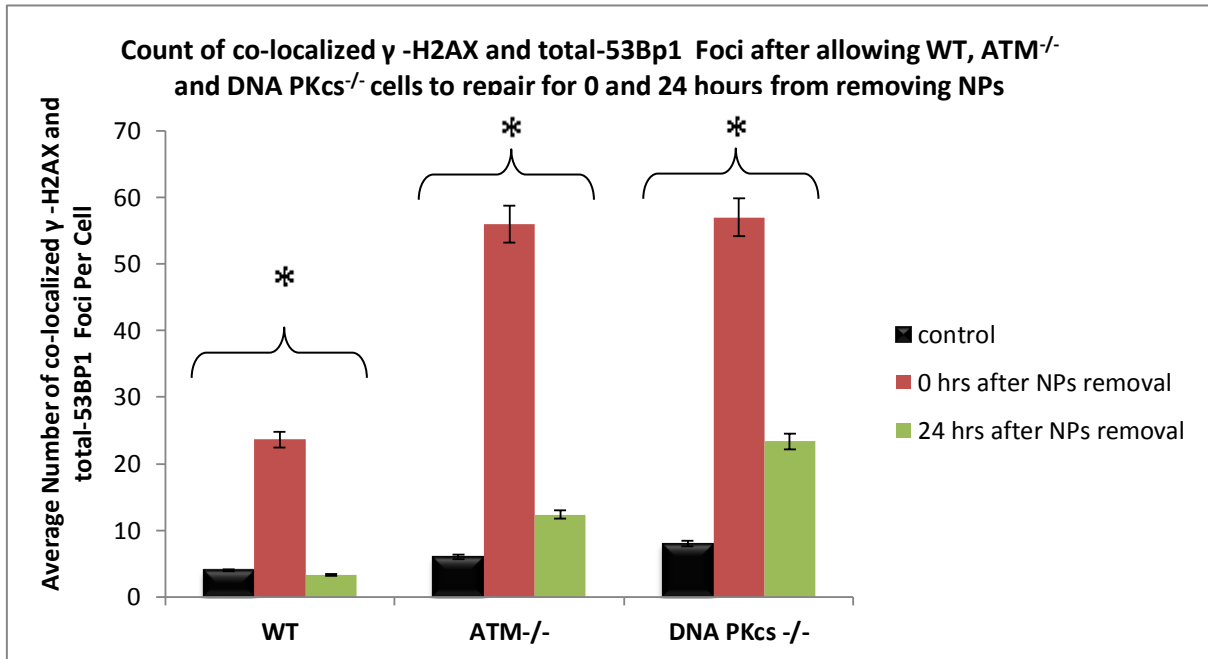


**Figure 14. immunofluorescent quantification of DNA double strand breaks in 1 Br hTERT cell line after 0 and 24 hours repair.** Representative graph showing the average number of co-localized  $\gamma$ -H2AX and total-53BP1 Foci per cell in 1 Br hTERT cell line after exposing the cells to 0.1  $\mu\text{g/ml}$   $\text{TiO}_2$  NPs for 24 hours and allowing the cells to repair for 0 and 24 hours after removal of the NPs. It was shown that there is a significant decrease between the number of co-localized  $\gamma$ -H2AX and total-53BP1 foci after 0 and 24 hours repair ( $P < 0.05$ ).

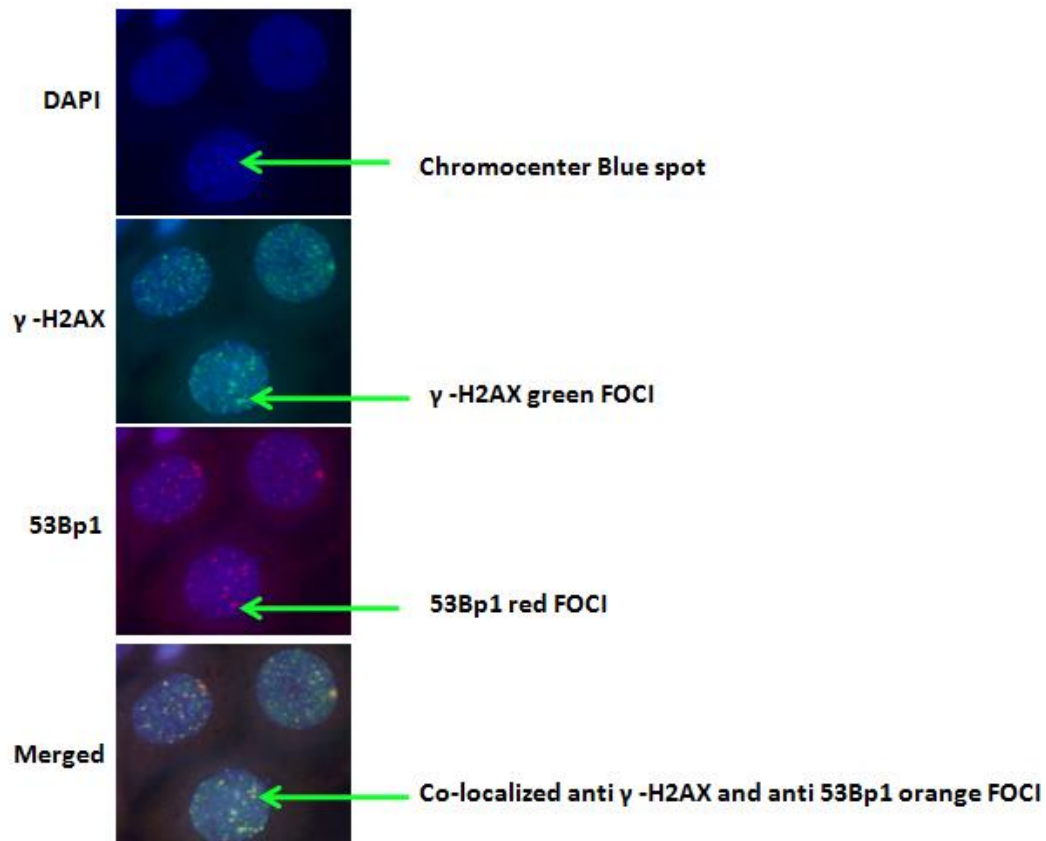
### **3.3.2 Immunofluorescent detection of DNA double strand breaks in WT, ATM<sup>-/-</sup>, and DNA PKC<sup>-/-</sup> cell lines.**

Since we observed significant genotoxicity in cancer as well as normal cells over acute exposure (24 hours) and no accumulation of DNA damage over prolonged exposure (168 hours) we decided to examine the repair of DNA repair which could have occurred in order to avoid increase in DNA damage over long exposure (168 hours). Consequently, immunofluorescent staining  $\gamma$ -H2AX and total-53Bp1 was carried out using confluent WT, ATM<sup>-/-</sup>, and DNA Pkcs<sup>-/-</sup> exposed to 0.1  $\mu$ g/ml TiO<sub>2</sub> NPs for 24 hours and allowed to repair in absence of NPs for 0 and 24 hours. This is because WT will confirm if normal cells will be able to repair the DNA damage induced by the NPs. While, ATM<sup>-/-</sup> and DNA Pkcs<sup>-/-</sup> will shed light into the role of DNA repair proteins that will be involved in repairing the DNA damage induced by the NPs. As a result WT cells showed significant decrease in DNA damage 24 hours after removing the NPs. While, ATM<sup>-/-</sup> cells showed significant decrease in DNA damage at euchromatin regions and persistent DNA damage at Heterochromatin regions. Whereas DNA Pkcs<sup>-/-</sup> showed significant decrease in DNA damage at heterochromatin regions and persistent DNA damage at euchromatin regions.





**Figure 15. Immunofluorescent quantification of DNA double strand breaks in WT, ATM<sup>-/-</sup>, and DNA PKC<sup>-/-</sup> cell lines. A.** Representative graph showing the Average number of co-localized  $\gamma$ -H2AX and total-53BP1 Foci per cell at 0 and 24 hours after removing the NPs and allowing WT, ATM<sup>-/-</sup>, and DNA PKcs<sup>-/-</sup> cells to repair. The three cell lines showed Significant rise in amount of co-localized  $\gamma$ -H2AX and total-53BP1 Foci between control and treated cells ( $P < 0.05$ ). However, 24 hours later after removing the Nanoparticles the three cell lines showed significant decrease in number of co-localized  $\gamma$ -H2AX and total-53BP1 Foci per cell ( $P < 0.05$ ).



**Figure 16. Immunofluorescent visualization of  $\gamma$ H2AX and 53BP1 in Mouse embryonic fibroblasts, MEFs.**

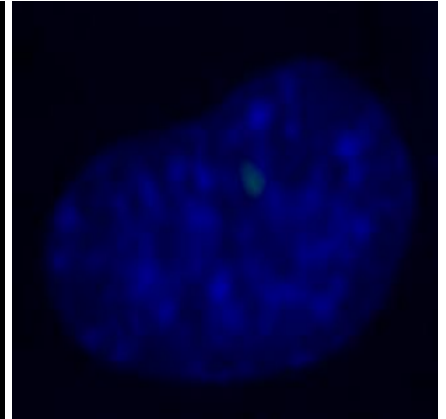
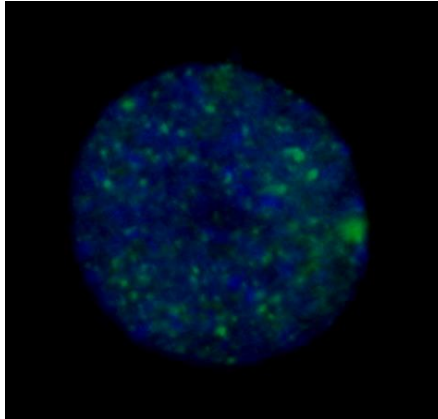
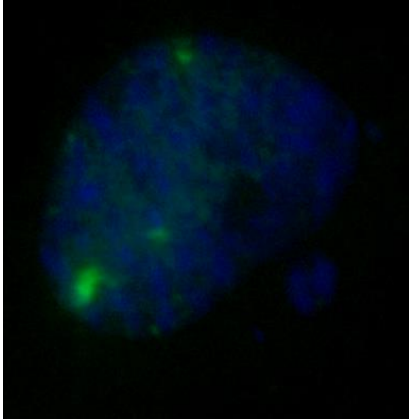
Representative 2-D images of WT MEFs nuclei were stained with DAPI (Blue Nuclei) and cells were stained for phosphorylated H2AX ( $\gamma$ H2AX) (green Foci) and total-53BP1 (red Foci).  $\text{TiO}_2$  NPs induces DSBs that forms  $\gamma$ H2AX and 53BP1 distinct foci that co-localized in the DAPI stained nucleus showing orange coloration.

Treated Nuclei 0 hours  
after removing  
Neocarzinostatin

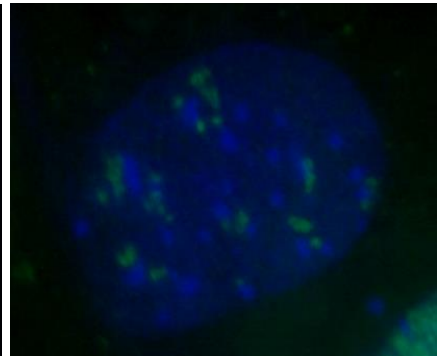
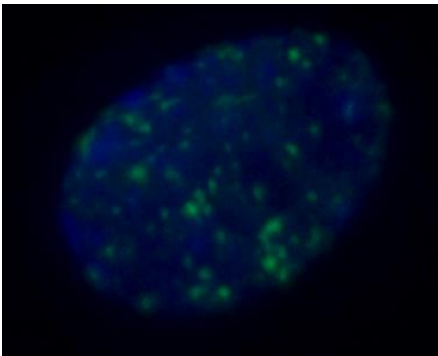
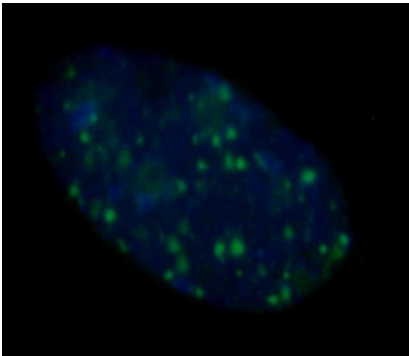
Treated nuclei 0 hours  
after removing NPs

Treated nuclei 24 hours  
after removing NPs

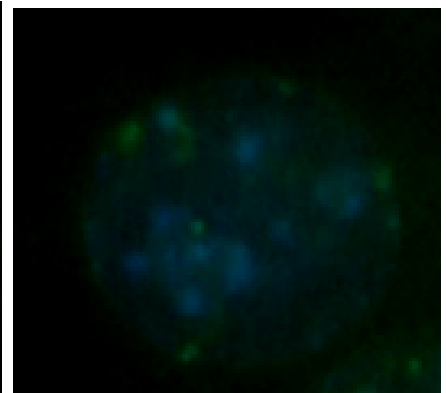
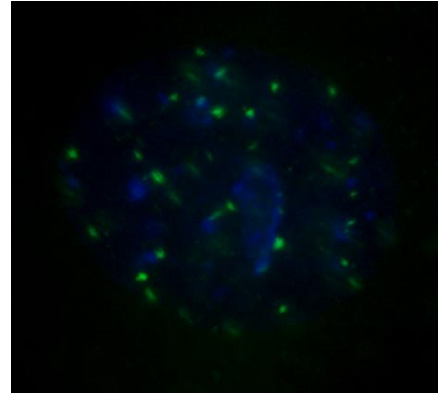
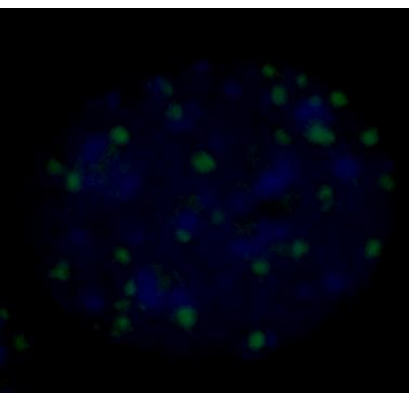
WT



ATM<sup>-/-</sup>



DNA PKC<sup>-/-</sup>



**Figure 17. Immunofluorescent Visualization of DNA double strand breaks in WT, ATM<sup>-/-</sup>, and DNA PKC<sup>-/-</sup> cell lines.** Representative 2-D images showing that DNA damage persists after 24 hours exposure to Neocarzinostatin (first column), NCS, which is known to be DSBs, is similar to DNA damage persists after 24 hours exposure to TiO<sub>2</sub> NPs (third column) indicating that the type of DNA damage induced by TiO<sub>2</sub> NPs is DSBs. WT cells showed lower number of foci than ATM<sup>-/-</sup> and DNA PKC<sup>-/-</sup> after 24 hours exposure to Neocarzinostatin or Titanium dioxide NPs reflecting the importance of ATM and DNA PKCs in repairing Damaged DNA induced by NCS or TiO<sub>2</sub> NPs. Also, the images showed that the DNA damage remained after 24 hours repair (Green spots) in ATM<sup>-/-</sup> cells were localized near or at the chromocenters of the genome (Blue spots). While DNA damage remained after 24 hours repair (green spots) in DNA Pkc<sup>-/-</sup> cells were localized away from chromocenters (Blue spots) and more at the Euchromatin regions of the genome (peripheral sides of the nuclei).

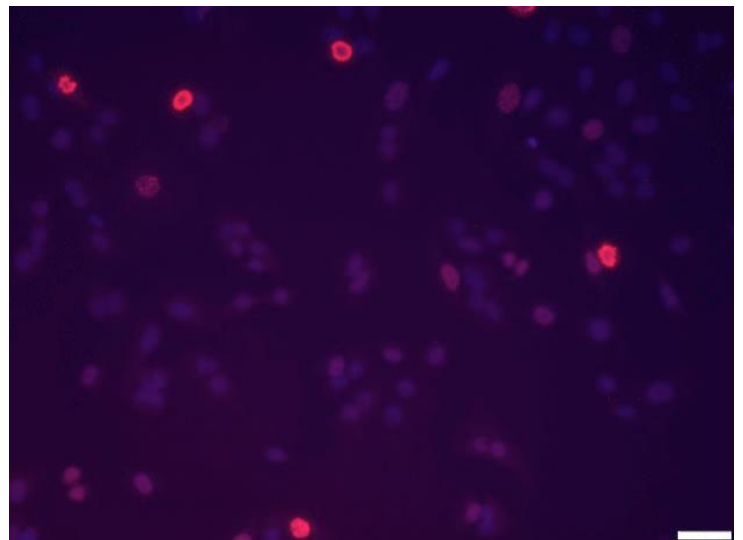
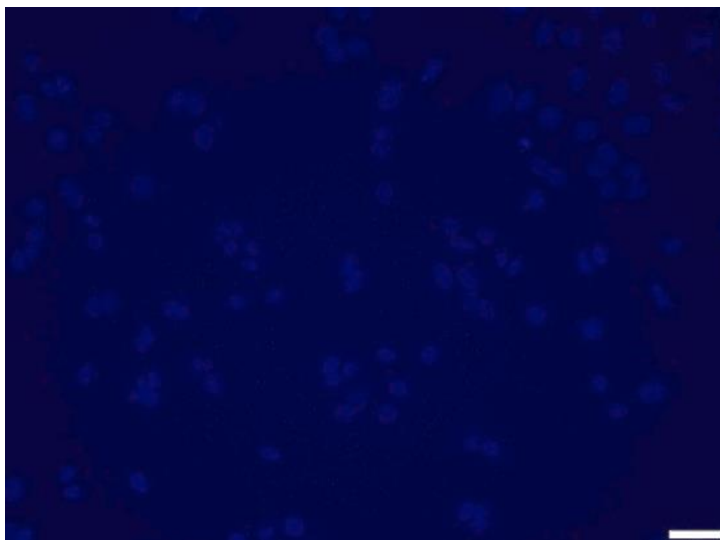
### **3.4 G2/M check point assay**

Since we showed significant genotoxicity induced by the NPs on normal, cancer and DNA repair deficient cells, we wanted to further examine the control of cell cycle checkpoint to prevent cells from entering mitosis in presence of damaged DNA and allowing the cells to repair. Thus, we decided to perform G2/M checkpoint assay to examine the efficiency of cell cycle control in each cell type after exposure to the NPs. As a result, U2OS, A549 and 1Br hTERT cells were exposed to different concentrations of TiO<sub>2</sub> NPs (0 - 0.1 - 0.5 - 100 - 200 µg/ml) for 24 hours. This was followed by immunofluorescent staining for Phospho-Histone H3. Results showed significant arrest in 1 Br hTERT at the lowest concentration of the NPs. while U2OS and A549 showed significant arrest only at the highest concentration of the NPs. Then we asked how long 1 BrhTERT cells will be able to maintain cell cycle checkpoint activation? On that note, we exposed 1 BrhTERT cells to 0.1 µg/ml TiO<sub>2</sub> NPs for 24 hours then cells were fixed and stained at different time points (0-4-8-12-24-48 Hours) after NPs removal. Results showed the cells were able to maintain arrest only for 12 hours after NP's removal. Moreover, WT, ATM<sup>-/-</sup> and ART<sup>-/-</sup> cells were exposed to (0.1 µg/ml) TiO<sub>2</sub> NPs for 24 hours then cells were fixed and stained at different time points (0-4-8-12-24-48 Hours) after NPs removal. ATM<sup>-/-</sup> cells (checkpoint deficient cells) showed no significant cell cycle arrest while WT and ART<sup>-/-</sup> showed significant arrest which was maintained in WT cells only for 12 hours while in ART<sup>-/-</sup> cells it was maintained for 48 hours. Quantification was carried out by calculating the average number of phosho-histone H3 positive cells per 100 cells.

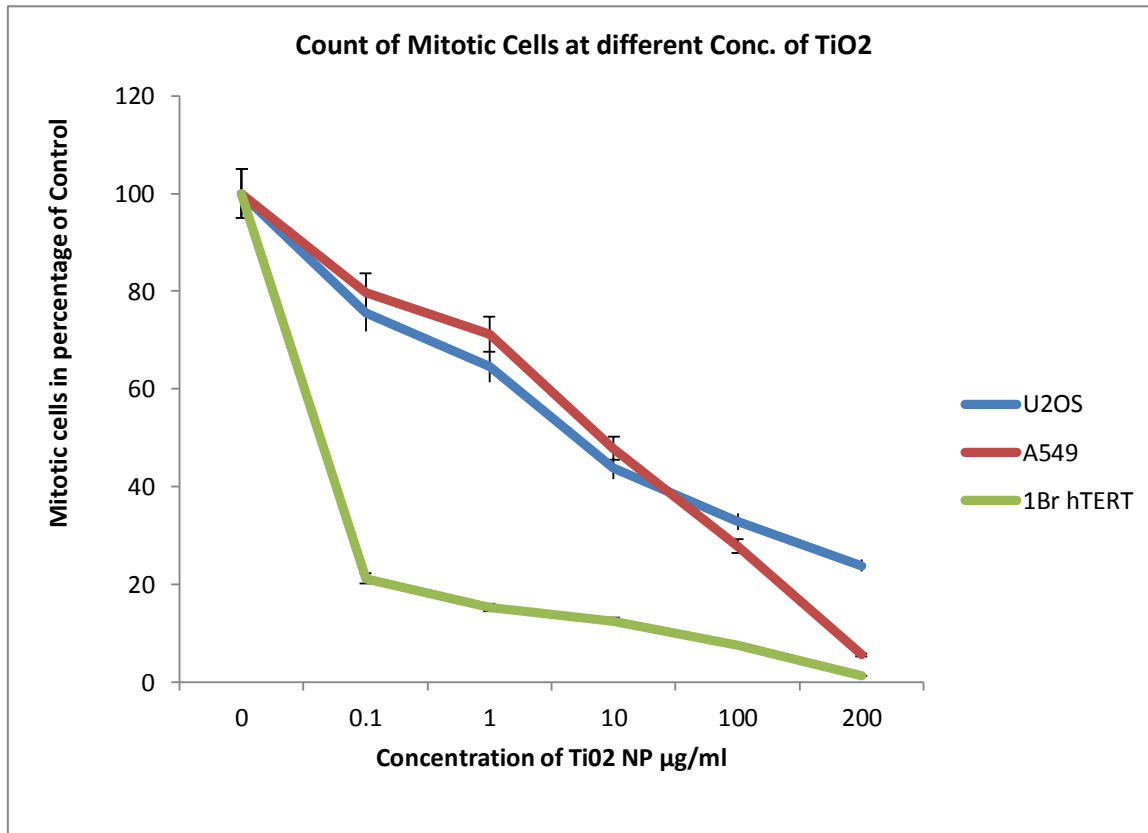
A

DAPI

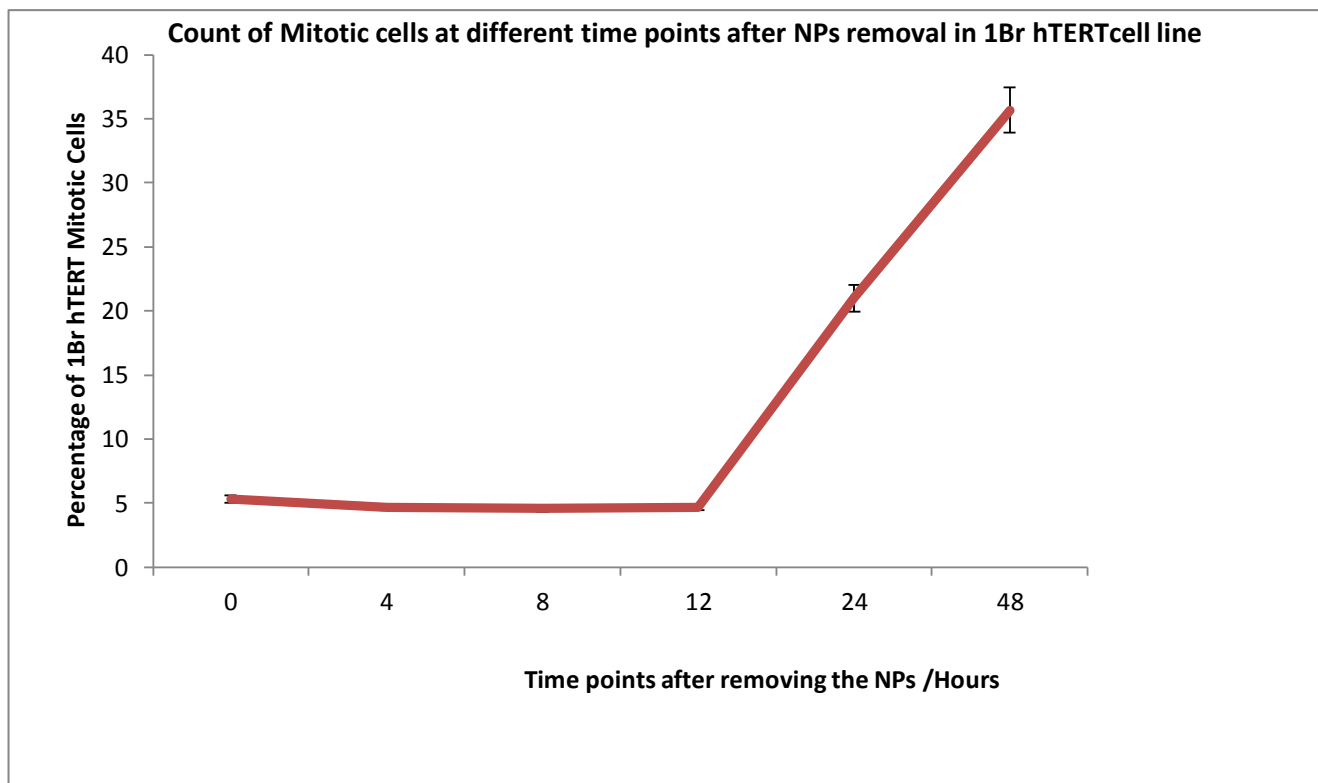
Merged



**B.**

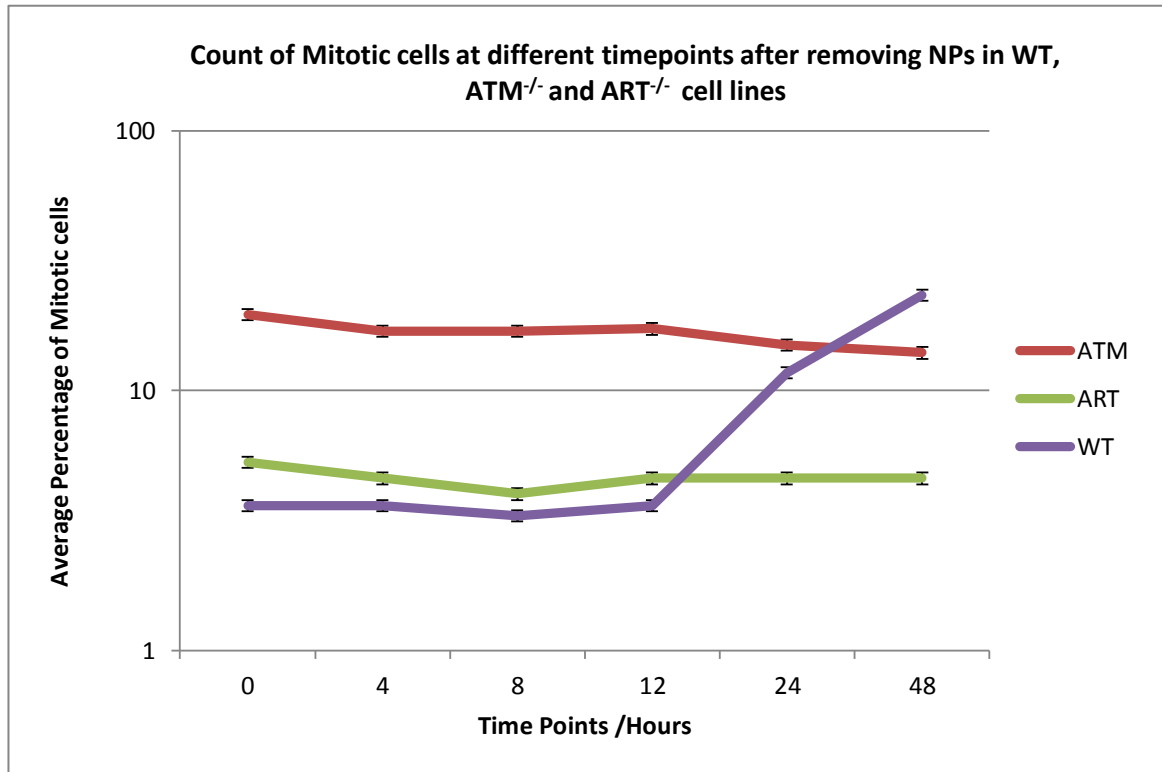


**Figure 18. Immunofluorescent detection of Mitotic cells in U2OS, A549 and 1Br hTERT cell lines exposed to different concentrations of NPs for 24 hours. A.** Representative images showing Nuclei stained with DAPI appear in blue and Mitotic cells stained with Phospho-histoneH3 appear in red. **B.** Representative graph showing significant negative correlation between the concentration of TiO<sub>2</sub> NPs and the percentage of Mitotic cells in percentage of control. Mitotic Fraction was quantified by counting the average number of Phospho-H3 positive cells per 100 cells in U2OS, A549 and 1Br hTERT cell lines exposed to different concentrations of TiO<sub>2</sub> NPs (0-0.1-1-10-100-200µg/ml) for 24 hours. The significance of cell cycle arrest was calculated for the three cell lines at the lowest concentration of TiO<sub>2</sub> NPs (0.1µg/ml). 1 Br hTERT cell line showed significant arrest at P <0.05 while A549 and U2OS cell lines showed no significant arrest at P <0.05.

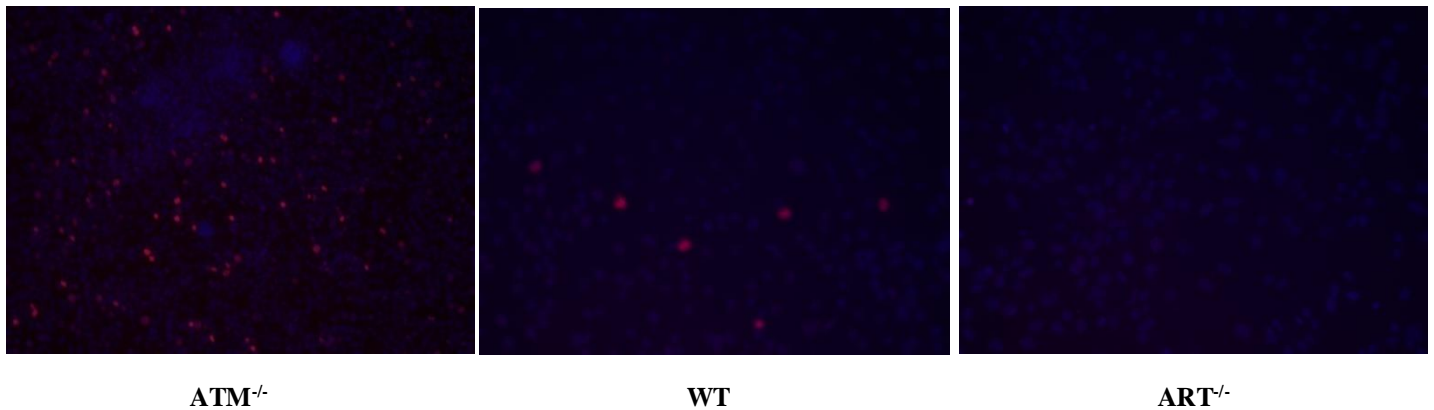


**Figure 19. Immunofluorescent detection of Mitotic cells in 1Br hTERT cell line that was fixed at different time points after removal of NPs** Representative graph showing the percentage of 1 Br hTERT Mitotic cells was calculated by counting the number of Phospho-H3 positive cell per 100 cells at different time points after removing the NPs (0-4-8-12-24-48 Hours). The cells were exposed to (0.1  $\mu$ g/ml) of the NPs for 24 hours then the NPs were removed and the percentage of Mitotic cells was calculated at different time points. 1 Br hTERT cells show significant cell cycle arrest for 12 hours after NPs removal but at 24 and 48 hours, significance increase in percentage of mitotic cells was observed at  $P < 0.05$

A



B.



**Figure 20. Immunofluorescent detection of Mitotic cells in WT, ATM<sup>-/-</sup>, and ART<sup>-/-</sup> cell lines that were fixed at different time points after removal of NPs. A.** Representative graph showing the percentage of Mitotic cells at different time points after removing the NPs (0-4-8-12-24-48 Hours). The cells were exposed to NPs (0.1µg/ml) for 24 hours then the NPs were removed and the percentage of Mitotic cells was calculated at different time points (0-4-8-12-24-48 Hours). ATM<sup>-/-</sup> cell line showed activated cell cycle while WT and ART<sup>-/-</sup> cell lines showed arrested cell cycle for 12 hours. Although ART<sup>-/-</sup> remained arrested up to 48hours, WT showed significant increase in percentage of Mitotic cells at 24 and 48 Hours (P<0.05). **B.** 2-D images show presence of Mitotic cells for ATM<sup>-/-</sup> and WT cell lines at 12 hours after removing the NPs while complete arrest was observed in ART<sup>-/-</sup> cell line at 12 hours after removing the NPs.



#### 4. Discussion

In the current study, we evaluated the impact of short- and long-term exposure to TiO<sub>2</sub> NPs on U2OS, A549 and 1Br hTERT cell lines. We reported that TiO<sub>2</sub> nanoparticles induce significant cytotoxicity at high concentration (200 µg/ml) over acute exposure, unlike prolonged exposure that showed no significant cytotoxicity (Figure 11), which are in line with the recent study (Armand et al., 2016). According to these cytotoxic data, we wondered if G2/M cell cycle check point failed to arrest cell division for long period of time in presence of DNA damage. As a result, cells start to divide in presence of DNA damage showing no significant cytotoxicity over prolonged exposure.

Therefore, we decided to use acute and prolonged exposure scenarios: high concentration over short period of time, low concentration over long period of time, respectively. This is to investigate the genotoxicity of TiO<sub>2</sub> NPs, to verify that on acute-exposure and prolonged exposure there will be DNA damage that will activate cell cycle checkpoint to arrest the cell for DNA repair. However, due to the fact that cancer cells such as U2OS and A549 are known to have defective DNA repair genes (Shi et al., 2013; Shirley et al., 2013), cells will not be able to repair the damage and prefer apoptosis showing significant reduction in cell survival at very high concentrations of TiO<sub>2</sub> NPs (Figure 11). While on prolonged exposure scenario DNA damage will be induced and maintained over long period of time that could lead to cell cycle checkpoint adaptation, where cell cycle G2/M check point fails to arrest the cell for a long period of time regardless the amount of DNA damage present within the cell. So G2/M checkpoint is released allowing the cells to enter M-phase in presence of damaged DNA. As a result, cells that showed significant decrease in viability after 24 hours they will show no significant decrease in viability after 1 week exposure (figure 11). This is because cells were not able to arrest the cell cycle for long period of time. Consequently, cells didn't have enough time to repair DNA damage or activate apoptosis. Thus, proliferation of genetically altered cells will occur which may escape recognition by more sensitive cell cycle checkpoints, leading to further carcinogenesis after long exposure to TiO<sub>2</sub> nanoparticles even at very low concentrations (Prolonged scenario).

On that note, we studied the genotoxicity of TiO<sub>2</sub> NPs by performing immunofluorescent staining for detection of DNA DSBs in U2OS, A549 and 1 Br hTERT after 24 -168 hours exposure to different concentrations of TiO<sub>2</sub> NPs (0-0.1-0.5-100-200µg/ml). This was performed by using anti-γ-H2AX and anti-total-53BP1. This is because upon induction of DSBs, sensor proteins MRE11-RAD50-NBS1, MRN complex, localizes ATM to the site of DSB. Simultaneously, auto phosphorylation of DNA-PKcs proceeds. Together, ATM and DNA-PKcs phosphorylates H2AX at serine 139 forming γ H2AX which will act as platform for recruitments of different proteins that will leads to recruitment of 53BP1 and activation of checkpoint for DNA repair functions. This shows that quantifying both γ-H2AX and 53BP1 foci will serve as distinctive biomarkers for quantifying the amount of double strand breaks per cell (Watters et al., 2009). As a result, we had demonstrated that acute and prolonged exposures were genotoxic in concentration dependent manner, increasing the concentration of TiO<sub>2</sub> NPs the average number of foci per cell significantly increases. Interestingly, we got No-Threshold model between the concentration of TiO<sub>2</sub> NPs and the number of

DNA DSBs per cell (Figure 13). This sheds light on titanium dioxide nanoparticles ability to have linear response with no safety threshold, unlike linear quadratic response. Also, we found that the genotoxic effect of NPs is time exposure independent; increasing time exposure there was no significant increase in genotoxicity. This reflects the stabilization of TiO<sub>2</sub> NPs intracellular accumulation over 1 week exposure. Thus, we can conclude that Humans can get harmed due to several small exposures (prolonged scenario) same as Humans exposed to one larger exposure (acute scenario) not due to cellular accumulation of NPs but possibly due to cellular adaptation.

Putting cytotoxic and genotoxic data together, they provides evidence that if long exposure maintains DNA DSBs, cell viability will not be reduced due to cellular adaptation. Consequently, we decided to confirm cellular adaptation by studying the progression of exposed cells in presence of DNA damage from G2 phase into mitosis using G2/M check point assay for acute and prolonged scenarios. So we investigated the progression of mitosis after exposing U2OS, A549 and 1Br hTERT cell lines to different concentrations of TiO<sub>2</sub> NPs (0-0.1-1-10-100-200µg/ml) for 24 hours by immunostaining phospho-histone H3, highly phosphorylated protein in Metaphase during mitosis (Goto et al., 1999). (Hendzel et al., 1997). This was followed by counting the number of phospho-histone H3 positive cells per 100 cells to calculate the Mitotic Fraction. As a consequence, we had showed that the higher concentrations of TiO<sub>2</sub> NPs have lower Mitotic fraction for the three cell lines (Figure 18) Most importantly, we found that the mitotic fraction was significantly decreased in 1 Br hTERT cells but no significant decline in mitotic fraction was detected in U2OS or A549 cells at the lowest concentration of TiO<sub>2</sub> NPs (0.1µg/ml). This clearly shows that normal cells as 1Br hTERT responded to smaller amount of DNA damage and activated cell cycle checkpoint at very low concentration of TiO<sub>2</sub> NPs. While cancer cells such as U2OS and A549 did not activate cell cycle checkpoint at very low concentration of TiO<sub>2</sub> NPs. This could reflect that normal cells are more sensitive to DNA damage as they have lower threshold of DNA damage than cancer cells to activate cell cycle checkpoint.

This raises a question: Will normal cells be able to maintain cell cycle checkpoint for a long period of time? To answer this question we decided to measure the mitotic fraction for 1 Br hTERT cells at different time points (0-4-8-12-24-48 hours) after removing the NPs (0.1µg/ml). We found that the cells were released from cell cycle checkpoint activation after 24 hours (figure 19). Therefore, we thought about one conclusion; normal cells were able to repair most of DNA damage and fall below the threshold of DNA damage needed to maintain G2/M checkpoint activation. To test this conclusion, we measured the amount of DNA damage remained after allowing 1 Br hTERT cells to repair for 0 and 24 hours after removing the NPs. Interestingly, we found that there was significant decrease between the amount of DNA damage at 0 and 24 hours from removing the NPs, and when we associated the total DNA damage in treated cells with untreated cells after 24 hours repair we found that there was no significant difference in number of DNA damage in treated cells than untreated cells (Figure 14). This clearly shows that the results supported our conclusion: the release of 1 Br hTERT cells from checkpoint activation was not due to cellular adaptation but due to successful DNA repair that decreases the amount of DNA damage per cell which allowed the release

of cells from cell cycle arrest. However on repeated exposure of NPs to cells there will be stabilization of the quantity of DNA damage per cell (Figure 13) leading to checkpoint adaptation which will allow the cell to be released and divide in presence of DNA damage as shown in cytotoxicity assay (Figure 11).

Now, that we demonstrated that genotoxicity of prolonged exposure scenario is not due to accumulation of DNA damage but it is due to cellular adaptation to arrest the cell cycle for long period of time in presence of constant amount of DNA damage. Then, there will be three possible fates the cell may undergo, either the cell will proceed with DNA damage through mitosis and cannot undergo cell division, Cytokinesis, forming multinucleated cell that leads to mitotic catastrophe or cell will undergo cell division and enter G1 where the master G1/S phase checkpoint will have lower threshold than G2/M phase arresting the cell due to presence of single double strand break (Löbrich & Jeggo, 2007). so the cells can either repair the DNA damage and divide or undergo apoptosis, or cells may also escape the master G1/S phase checkpoint and divide in presence of DNA damage which will leads to genetic instability (Huang et al., 1996); (Mc Gee, 2015).

Since we confirmed the necessity of activating and maintaining G2/M cell cycle checkpoint for DNA damage to be repaired before cell division to prevent carcinogenesis, we wanted to further study the molecular mechanisms involved in activating and maintaining G2/M checkpoint. As a result, the mitotic fraction for ATM<sup>-/-</sup>, ART<sup>-/-</sup>, and WT cells was measured. The three cell lines were exposed to 0.1µg/ml of TiO<sub>2</sub> NPs for 24 hours then at different time points after removing NPs (0-4-12-24-48 Hours) the number of phospho-histone H3 positive cells per 100 cells was counted. Consequently, it was shown that ATM<sup>-/-</sup> cells failed to activate cell cycle checkpoint and arrest Mitosis at any time point. While, ART<sup>-/-</sup> and WT cells were able to activate cell cycle checkpoint and arrest the cell (Figure 20). This clearly shows the role of ATM in activating cell cycle checkpoint to arrest the cell cycle for DNA repair. This is because checkpoint deficient cells such as ATM<sup>-/-</sup> cells failed to arrest the cell cycle while the checkpoint proficient cells such as WT and ART<sup>-/-</sup> succeeded to activate cell cycle checkpoint and arrest the cell cycle for DNA repair. More interesting, it was observed that WT cells released from checkpoint activation after 24 hours from removing the NPs while ART<sup>-/-</sup> remained arrested up to 48 hours (Figure 20). This sheds light on the threshold of DNA damage that the cells have to fall below in order to be released from checkpoint activation. This is because WT cells have normal DNA repair genes that repaired most of the DNA damage allowing the cells to fall below the threshold of DNA damage and release from checkpoint activation. On the other hand, ART<sup>-/-</sup> cells were arrested for longer time as they are Artemis deficient cells, nuclease needed for DNA repair by cNHEJ, so they were not able to repair the DNA damage and stayed above the threshold of DNA damage resulting in arresting the cells for longer period of time. Thereby, we were able to prove that there is threshold of DNA damage the cells need to go beyond in order to activate G2/M checkpoint. This is because it was observed that there is a significant decrease in the amount of DNA damage after 24 hours repair in WT cells, like 1Br cells (Figure 15). This indicates that the cells were released from cell cycle checkpoint activation due to decrease in the total amount of DNA damage which make the cells fall below the threshold of DNA damage

needed to activate and maintain cell cycle G2/M checkpoint. Moreover, we were able to show that the threshold of DNA damage was not a single DNA DSB (10-20DSBs). This is because we measured the amount of DNA damage remained unrepaired at the time of checkpoint release; there was more than single DNA damage per cell (Figure 15) Also it was observed that the amount of DSBs remained at time of checkpoint release did not exceed the threshold of DNA damage for G2/M checkpoint activation (10-20 DSBs) that was demonstrated by (Löbrich & Jeggo, 2007)

Given that we were able to show that TiO<sub>2</sub> NPs are genotoxic, causing the most biologically dangerous DNA damage, DNA double strand breaks, we became more interested in studying the repair pathways that cells will choose to repair the DNA damage induced by the NPs. As mentioned above, DNA double strand breaks repaired in fast or slow kinetics depending on the position of DNA double strand breaks (Goodarzi et al.). So if the DNA double strand breaks occurs near or at the heterochromatin region it will need ATM signaling pathway that will relax the heterochromatin region to facilitate DNA repair (slow kinetics) (Goodarzi et al.). On the other side, if DNA DSBs occur away from heterochromatin region then DNA repair will occur immediately without need of ATM relaxation (fast kinetics) then complete DNA repair in fast or slow kinetics is achieved through c-NHEJ or HR depending on the phase of the cell cycle (Iliakis et al., 2015). As mentioned above, c-NHEJ repair is predominant in all phases while HR is restricted to S or G2 phases only (Dexheimer, 2013). Consequently we decided to investigate the location of DNA lesions induced by TiO<sub>2</sub> NPs to G1 phase synchronized cells. In order to analyze which repair kinetics and pathway will repair the DNA lesions, we decided to visualize the position and count the number of  $\gamma$ -H2AX Foci in WT, ATM<sup>-/-</sup> and DNA PKC<sup>-/-</sup> cell lines that were synchronized in G1 phase, cells were allowed to reach 100% confluency before exposure to NPs. This was followed by exposing the three cell lines to 0.1  $\mu$ g/ml TiO<sub>2</sub> NPs for 24 hours. Then immunofluorescent staining for detection of DNA double strand breaks using anti- $\gamma$ -H2AX and anti-total-53BP1 was performed. This is because WT will serve as positive control cells. ATM<sup>-/-</sup> cells will show if any of the DNA damage lesions will need ATM signaling pathway to promote chromatin relaxation to allow DNA repair at the heterochromatic region. DNA PKCs<sup>-/-</sup> cells will show if the NPs causes DNA double strand breaks at regions other than heterochromatin e.g. euchromatin. This is because if the DNA DSBs occur at the euchromatin it will repair in fast kinetics using c-NHEJ repair pathway that will requires the recruitments of DNA PKCs on the opposing double strand break ends promoting the tethering of the two DNA ends and allowing the two DNA termini become accessible by c-NHEJ (Dexheimer, 2013).

Interestingly, we found that the three cell lines have  $\gamma$ -H2AX foci at the distinctive regions called “chromocenters”, which refers to Pericentric and centomeric heterochromatin, as well as euchromatin regions (Figure 17). Next, we wanted to examine the efficiency of each cell to repair DNA damage at both heterochromatin and euchromatin regions. Thus, we allowed the three cell lines WT, ATM<sup>-/-</sup> and DNA PKCs<sup>-/-</sup> to repair for 24 hours after removal of NPs. This was followed by immunofluorescent staining for detection of DNA double strand breaks using anti- $\gamma$ H2AX and anti-total-53BP1. Consequently, WT cells shows DNA repair at most of the DNA DSBs. While, ATM cells showed that most of the DNA DSBs those were at the euchromatin were repaired but the

DNA DSBs those were near or at the chromocenters were unrepaired. This confirms the results that were shown before by (Goodarzi et al.) DNA DSBs at heterochromatin region need ATM signaling to promote chromatin relaxation for DNA DSBs repair. Thus, in absence of ATM, DNA DSBs at heterochromatin caused by the NPs could not be repaired. This clearly shows that the repair at heterochromatin region is ATM dependent. Moreover, we observed significant lower amount of DNA DSBs remained after 24 hours of DNA repair in ATM<sup>-/-</sup> compared to DNA PKcs<sup>-/-</sup> cells. So we decided to calculate the percentage of DNA damage remained in absence of ATM after allowing cells to repair for 24 hours. As a result, it was shown that only 21% of the total DNA damage remained unrepaired in ATM<sup>-/-</sup> cells. This confirms the results that were shown by (Goodarzi et al.), that the amount of DNA damaged to be repaired by ATM signaling pathway at heterochromatin region is not more than 25% of the total DNA DSBs. Also, it was shown that the percentage of unrepaired DNA damage in absence of ATM corresponds to the amount of the heterochromatin region within genome (20-30%) (Goodarzi et al.) Thus, our results strengthen the role of ATM in repairing DNA DSBs caused by the NPs at the heterochromatin region.

Whereas, DNA PKcs<sup>-/-</sup> showed that most of the DNA DSBs near to the chromocenters were repaired while DNA DSBs at the euchromatin were unrepaired. It was expected that the DNA DSBs at the euchromatin will not be repaired in absence of PKcs. As PKcs is needed to allow DNA repair by c-NHEJ in G1 phase (Iliakis et al., 2015). However, we found that DNA DSBs at heterochromatin region was repaired in absence of PKcs in G1 phase. Consequently, two questions were raised: How heterochromatin regions repaired DNA DSBs in absence of c-NHEJ in DNA PKcs<sup>-/-</sup> cells in G1 phase? Why heterochromatin regions only succeeded to repair DSBs while euchromatin region had persistent DNA damage in absence of c-NHEJ in G1 phase?

To answer the two questions, it was shown by recent studies (Iliakis et al., 2015) that the cell utilizes “backup” repair pathway such as alt-EJ repair pathway to remove any unprotected DNA ends in existence of pre-enzymatic activities. Thus, whenever the main pathway either c-NHEJ or HRR, fails to repair the DSBs, alt-EJ will take place as a backup to protect the damaged DNA at any price. This could explain why in absence of DNA PKcs in G1 phase, cells utilizes alt-EJ repair pathway to repair DNA DSBs at heterochromatin after relaxation promoted by ATM. However, alt-EJ repair pathway was not utilized by the cell to repair DSBs at euchromatin regions. This is also could strengthen the proposed hypothesis: DNA damage at active regions of genome such as euchromatin will persists, and DNA clustering at these damaged regions will occur to sequester DSBs until more appropriate cell cycle phase is reached for DSBs to be repaired by error free repair pathway such as HR in S-or G2 phase (Aymard et al., 2017). This is because repairing active genes at euchromatin regions by error prone repair pathway such as alt-EJ will be so detrimental for the cell, due to increase risk of mutation and chromosomal translocation within this repair pathway.

## 5. Conclusion

In conclusion the experiments carried out in this study illustrated that prolonged exposure to low concentration (0.1µg/ml) of Titanium dioxide nanoparticles induces significant genotoxicity in treated cells compared to untreated cells. Thus, on prolonged exposure to low concentration of TiO<sub>2</sub> NPs cells will be arrested due to significant genotoxicity.

Also, it was observed that in order to activate cell cycle G2/M checkpoint, cell has to exceed threshold of DNA damage. Consequently, cell must fall below this threshold of DNA damage in order to be released from cell cycle G2/M checkpoint. Interestingly, it was shown that the threshold of DNA damage the cell has to fall below in order to be released from arrest is not a single DSB. However, we showed that G2/M checkpoint is a negligent checkpoint that will fail to arrest the cell cycle for long period of time and cells will be released from arrest without falling below the threshold of DNA damage and enter Mitosis with significant amount of DNA DSBs leading to genetic instability.

While studying the genotoxicity of the NPs we found that the type DNA damage induced by the Nanoparticles we used in this study was the most biologically hazardous DNA damage, Double strand breaks. Also, DNA DSBs induced by NPs were observed at heterochromatin as well as euchromatin regions of the genome. Finally, it was shown that DNA DSBs repair in G1 phase at heterochromatin region is ATM<sup>-/-</sup> dependent and DNA PKcs<sup>-/-</sup> independent. While DNA DSBs repair at euchromatin region is DNA PKcs dependent and ATM independent.



## References.

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